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ADAPTATION BY MICROBIAL POPULATIONS

Norman C. Dondero, et al

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WP-00645-03

ADAPTATION BY MICROBIAL POPULATIONS

NORMAN C. DONDERO, PRINCIPAL INVESTIGATOR

AND

T.B.S. PRAKASAM, RESEARCH ASSISTANT

FOR THE PERIOD SEPT. 1, 1965 TO JAN. 15, 1966

DEPARTMENT OF ENVIRONMENTAL SCIENCES,
RUTGERS UNIVERSITY, NEW BRUNSWICK, N. J.

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T.B.S. Prakasam, Research Assistant

For the Period Sept. 1, 1965 to Jan. 15, 1966

Prepared: Jan. 15, 1966

SUMMARY:

1. Activated sludge extracts were superior to other culture media for obtaining the maximal viable counts of the aerobic heterotrophic bacteria of activated sludges. The extracts of activated sludges from different treatment plants differed in their ability to evoke maximal counts.

2. The replica plate technique was studied as a tool for characterizing bacterial populations as found in activated sludge, taking into account sources of inherent error: (1) failure of transfer of colonies from master plates, (2) impure colonies, and (3) syntrophism and antibiosis.

A minimum of 92.6% replication was achieved at the 0.05 significance level with five replications from a master plate, and a maximum of 12% of the total were mixed colonies in any experiment.

For determining the quantitative distribution of nutritional types of aerobic bacteria in activated sludge, the replica plate method was reliable to about 88% at the 0.05 significance level.

3. Forty percent of the bacterial colonies grown on activated sludge extract agar did not grow on an agar containing glucose, amino acids, growth factors, and salts, suggesting that autogenous growth factors may be produced by activated sludge which are required by a substantial segment of the population.

4. Using C^{14} labelled sorbitol with glucose, sequential removal of the compounds did not occur significantly in treatment plant activated sludge; however, in an artificially developed sludge, which did not have the population heterogeneity of treatment plant activated sludge, sequential removal occurred.

5. An activated sludge developed on glucose, nutrient broth, and salts degraded a variety of aromatic compounds, but in a different fashion than did treatment plant sludge. The patterns of degradation of the compounds suggested that Stanier's simultaneous adaptation pattern did not apply to the mixed populations studied. The sludge from the synthetic waste differed from treatment plant activated sludge in total numbers as well as in numbers of bacteria able to grow on various aromatics. In the process of adapting to utilization of aromatic substrates, the populations of bacteria became differentiated from each other.

Introduction: One of the simplest ways to evaluate the adaptation made by a population is to compare its structure and activities before and after reaction to environmental stimuli or stresses.

In order to evaluate by enumeration of species, it is necessary to be able to recognize and differentiate the individuals of the species. This is particularly a difficult task with bacteria, not only because of the great labor involved in the isolation of pure cultures and the uncertainties of identification and taxonomy, but also because there are no 'non-selective' culture media of proven or acknowledged composition to permit the full enumeration of the majority of different types of microorganisms in activated sludge, sewage or water.

A further apparent complication in studying adaptation by microbial populations is that the most useful technic for enumeration and cultivation, the dilution plate counting method, magnifies the errors inherent in sampling by eliminating from consideration the most important elements which contribute to the flexibility of microbial populations, the microorganisms which are present in small numbers but which may, given the opportunity, become the dominant elements of the biocoenosis.

The methods of surmounting the obstacles for bacterial populations are described in this report. The studies have been confined to the aerobic heterotrophic bacteria of activated sludge, i.e., those most active in the aerobic destruction of organic matter. Fortunately, the enumeration and classification of the bacteria need not be taxonomic and is, perhaps, more significant when based on physiological and nutritional characteristics. A system for meeting these problems in soil microbiology had already been devised by Lochhead and his students in Canada, and experimental work for the evaluation of a productive, 'non-selective' medium, i.e., activated sludge extract, analogous to Lochhead's soil extract, is described in this report. Furthermore, Lochhead's series of mutually exclusive, nutritionally complex media were tested.

To overcome the discouraging labor of isolating, purifying, and testing each colony on a large number of culture media, the replica plating technic was studied and found to be acceptable in spite of some inherent drawbacks.

The problem of evaluating the minor elements of the population have been postponed pending the solution of the more pressing problems at hand.

Currently, the analyses of the results obtained in the population studies of laboratory-grown activated sludges are being processed by statistical considerations to get a cognizance of the ecological picture. Methods of analysis of populations as described in Slobodkin (1962), Hazen (1964), and Andrewartha (1961) are also being considered.

ENUMERATION OF AEROBIC HETEROTROPHS IN ACTIVATED SLUDGE.

Cultural studies of the general flora of activated sludge require a medium as non-selective as can be accomplished. The general approach made to meet this aim was similar to the one reported for soil micro-organisms (Lochhead⁴⁸) and bacteria of the marine environment (Skerman '63). 172

In the present investigation, culture media for obtaining maximal bacterial counts of activated sludge and sewage were evaluated. In these studies, the viable counts of settled sewages and activated sludges from three treatment plants were compared on several agar culture media. Activated sludge extract used in these studies was prepared by autoclaving the mixed liquor from a treatment plant and separating the extract by filtration through glass wool. The composition and preparation of the culture media listed are given in the appendix:

1. activated sludge extract (single strength)
2. activated sludge extract (double strength)
3. activated sludge extract + soil extract
4. sewage agar
5. iron-peptone agar
6. basal medium
7. basal medium + yeast extract
8. Taylor's medium
9. modified Taylor's medium
10. soil extract
11. nutrient agar (standard meat extract-peptone)

In the following descriptions of plating experiments, the activated sludge extract (ASE) agars were inoculated with portions of the same sample of activated sludge from which the extracts were prepared. Preparation of activated sludge extract media and plating of activated sludge on different media was done as quickly as possible after collection of activated sludge samples.

Presentation of Results in Plating Experiments: Since bacterial viable plate counts follow the Poisson distribution, the use of statistical procedures designed for normally distributed populations is not legitimate in analyzing the plate count data. To normalize the distribution of plate counts, square root transformation was done. This transformation involves the taking of the positive square root of each plate count (x). The means of plate counts reported in the results of plating experiments were obtained by squaring the arithmetic mean of all the $\sqrt{x}$ and not by averaging the plate counts as such. Analysis of variance (Dixon and Massey, 1957) was done on the square-root transformed data. The lsd (least significant difference) (Steel and Torrie, 1960) was derived

from the transformed data and was used in making decisions as to which differences in means are significant and which are not. A typical statistical analysis of the plate count data was illustrated by an example in the appendix using small integers. Also included in the appendix is an analysis of a set of typical plate count data.

Plating Experiment I. A sample of Marlboro activated sludge was plated on the following media: sewage agar, ASE + soil extract, ASE (single strength and double strength), Taylor's medium, basal medium, basal medium + yeast extract and nutrient agar. Table 1(a) represents the results obtained. Analysis of variance of the transformed data indicated that there was a significant difference between the mean counts of several different media. Lsd was employed to judge the differences in means and Table 1(b) indicates the grouping of media according to productivity, i.e. according to highest viable counts. The most productive media were the ASE agar (single and double strength) and Taylor's medium.

Plating Experiment II. Marlboro activated sludge was plated on iron-peptone agar (Ferrer, Stapert & Sokolski, 1963), and casitone-yeast extract agar, since these were found to be good culture media for certain water bacteria and for certain sewage bacteria. Plates containing ASE medium as well as nutrient agar were also plated simultaneously for comparison. Tables 2(a) and 2(b) represent the results.

Table 2(a). Viable Counts of Marlboro Activated Sludge.
Plating Experiment II.

No.	Medium	Mean Count (10^6 /ml)
1	ASE agar	120.56
2	iron-peptone agar	24.80
3	casitone-yeast extract	18.40
4	nutrient agar	23.04

Table 1 (a). Viable Counts of Marlboro Activated Sludge. Plating Experiment I.

<u>Medium</u>	<u>Mean Count (10^6/ml)</u>
1. Sewage agar	78.32
2. Activated Sludge extract + soil extract	68.39
3. Activated Sludge extract (Single strength)	152.52
4. Activated Sludge extract (double strength)	175.56
5. Taylor's Medium	139.24
6. Basal Medium	46.65
7. Basal Medium + Yeast ext.	61.93
8. Nutrient agar	43.29

Table 1 (b). Effectiveness of Culture Media for Supporting High Viable Counts. (1)

Medium No.	8	6	7	2	1	5	3	4
Mean Count	43.29	46.65	61.93	68.39	78.32	139.24	152.52	175.56

(1) Media of equal effectiveness are ranked together by a bar.

Table 2(b). Effectiveness of Culture Media for Supporting High Viable Counts.

Rank*	2	2	2	1
Medium No.	3	4	2	1
Mean count (10^6 /ml):	18.4	23.04	24.80	120.56

* Rank 1 = highest productivity

Analysis of variance of the data of experiment II showed that the means were significantly different. By ranking with lsd test nutrient agar, iron-peptone medium, and casitone-yeast extract medium did not differ significantly in their productivity, but ASE (Marlboro) was superior.

Plating Experiment III. In another experiment in which Marlboro activated sludge was plated, the results (Table 3) also indicate that ASE (single strength) gave higher counts than other media; however, there was a lower value on ASE (double strength).

Table 3. Viable Counts of Marlboro Activated Sludge. Plating Experiment III.

No.	Medium	Mean Count (10^6 /ml)
1	Activated sludge ext. + soil extract	66.1
2	ASE (single strength)	103.0
3	ASE (double strength)	54.6
4	Soil extract	53.6
5	Taylor's medium	67.4
6	Nutrient agar	33.4

The suppression of total counts on these plates appeared to result from antagonistic effects of several large colonies of bacteria which suppressed the growth of other colonies. This particular observation, though not encountered in several other plating experiments is presented here to indicate that higher concentrations of nutrients in the activated sludge extract can limit the growth of some types by stimulating antagonistic colonies permitting the growth of only a few resistant types.

Plating Experiment IV. Two sets of homologous plating experiments (i.e., plating bacteria on extracts of sewage or activated sludge which originated from the same treatment plant) were conducted with samples of sewage and activated sludge of the Bernardsville, N.J., treatment plant. Table 4(a) gives the results, and a summary of ranking by 1st in order of productivity is presented in Table 4(b).

Table 4(a). Viable Counts of Bernardsville Sewage and Activated Sludge. Plating Experiment IV.

No.	Medium	Run 1		Run 2	
		Sewage	Act. Sludge	Sewage	Act. Sludge
		Mean Count (10 ⁶ /ml)	Mean Count (10 ⁶ /ml)	Mean Count (10 ⁶ /ml)	Mean Count (10 ⁶ /ml)
1	Sewage agar	4.33	104	12.1	67.80
2	soil ext.	0.96	1.69	7.8	68.56
3	ASE	3.31	136.9	14.67	92.16
4	Taylor's Medium	11.02	380.3	13.18	110.30
5	Nutrient agar	5.85	104	13.54	100

Analysis of the data indicated that the mean counts on several different media were significantly different. Although some of the counts were occasionally high, as in Taylor's medium, ASE agar gave appreciably and consistently better results with different samples.

Table 4(b). Effectiveness of Culture Media for Supporting High Viable Counts of Sewage and Activated Sludge. Plating Experiment IV.

		<u>Run 1</u>					<u>Run 2</u>				
		<u>Sewage</u>					<u>Activated Sludge</u>				
Rank ¹		5	4	3	2	1	3	2	2	2	1
Medium No.		2	3	1	5	4	2	1	5	3	4
Mean count (10^6 /ml)		0.96	3.31	4.33	5.85	11.02	1.69	104	104	136.9	380.3
- 7 -											
Rank ¹		4	3	2	2	1	3	3	2	2	1
Medium No.		2	1	4	5	3	1	2	3	5	4
Mean count (10^6 /ml)		7.8	12.17	13.18	13.54	14.67	67.8	68.56	92.16	100	110.3

¹ Rank 1 = highest productivity

To test whether there would be differences in viable counts by plating activated sludge on an ASE medium made from another treatment plant, the following heterologous plating studies were undertaken.

Plating Experiment V. Bernardsville activated sludge was plated on Marlboro ASE agar as well as on other media. Table 5 represents the results obtained.

Table 5. Viable Counts of Bernardsville Activated Sludge.
Plating Experiment V.

No.	Medium	Mean Count (10^6 /ml)
1	Soil extract	31.14
2	Sewage agar	112.54
3	ASE (Bernardsville)	222.00
4	ASE (Marlboro)	543.00
5	Taylor's medium	156.30
6	Nutrient agar	265.70

The heterologous plating of Bernardsville activated sludge on Marlboro ASE yielded a significantly higher count. This increase could have been due to the following possibilities: (1) Marlboro sludge contained nutrients absent from other substrates, (2) Bernardsville sludge extract contained inhibitors not present in Marlboro sludge extract, or (3) other unrecognized effects resulting from differences in received waste or in plant operation.

Plating Experiment VI. Homologous and heterologous plating of sewage and activated sludge from the Morristown plant was done. Two other media were also used. The results are presented in Table 6.

Table 6. Viable Counts of Morristown Sewage and Activated Sludge. Plating Experiment VI.

No.	Medium	Sewage	Activated Sludge
		Mean Count (10 ⁶ /ml)	Mean Count (10 ⁶ /ml)
1	Sewage agar (Morristown)	2.13	49.98
2	Sewage agar (Marlboro)	3.24	98.80
3	ASE (Morristown)	2.37	69.22
4	ASE (Marlboro)	4.41	201.60
5	Taylor's medium	2.96	73.10
6	Nutrient agar	4.24	64.20

The results indicate that the Marlboro ASE medium was superior in productivity to other plating media.

Plating Experiment VII. In this experiment, the Taylor's medium was modified by substituting activated sludge extract for soil extract to ascertain whether the viable counts on the modified medium would be higher than on ASE media. To avoid bias, samples of sewage and activated sludge were subjected to homologous as well as heterologous plating on the same day, i.e., each sample was plated on all the media under consideration. In plating sewage, sewage agar was used in addition to other media, but in plating of activated sludge, sewage agar was omitted since it had not previously given higher counts. All samples of sewage and activated sludge were collected from the three treatment plants on the same day and plates of ASE as well as of sewage agar were made from these samples. Small quantities of each of the sewages and activated sludges were saved for plating inocula. Plating of activated sludges and sewages was done on the day of sample collection to avoid effects incidental to storage. The results of the plating study are presented in Figures 1 and 2. Tables 7(a) and 7(b) indicate the order of productivity of the various media as determined by lsd.

FIG. 1. PLATE COUNTS OF ACTIVATED SLUDGES
ON DIFFERENT MEDIA

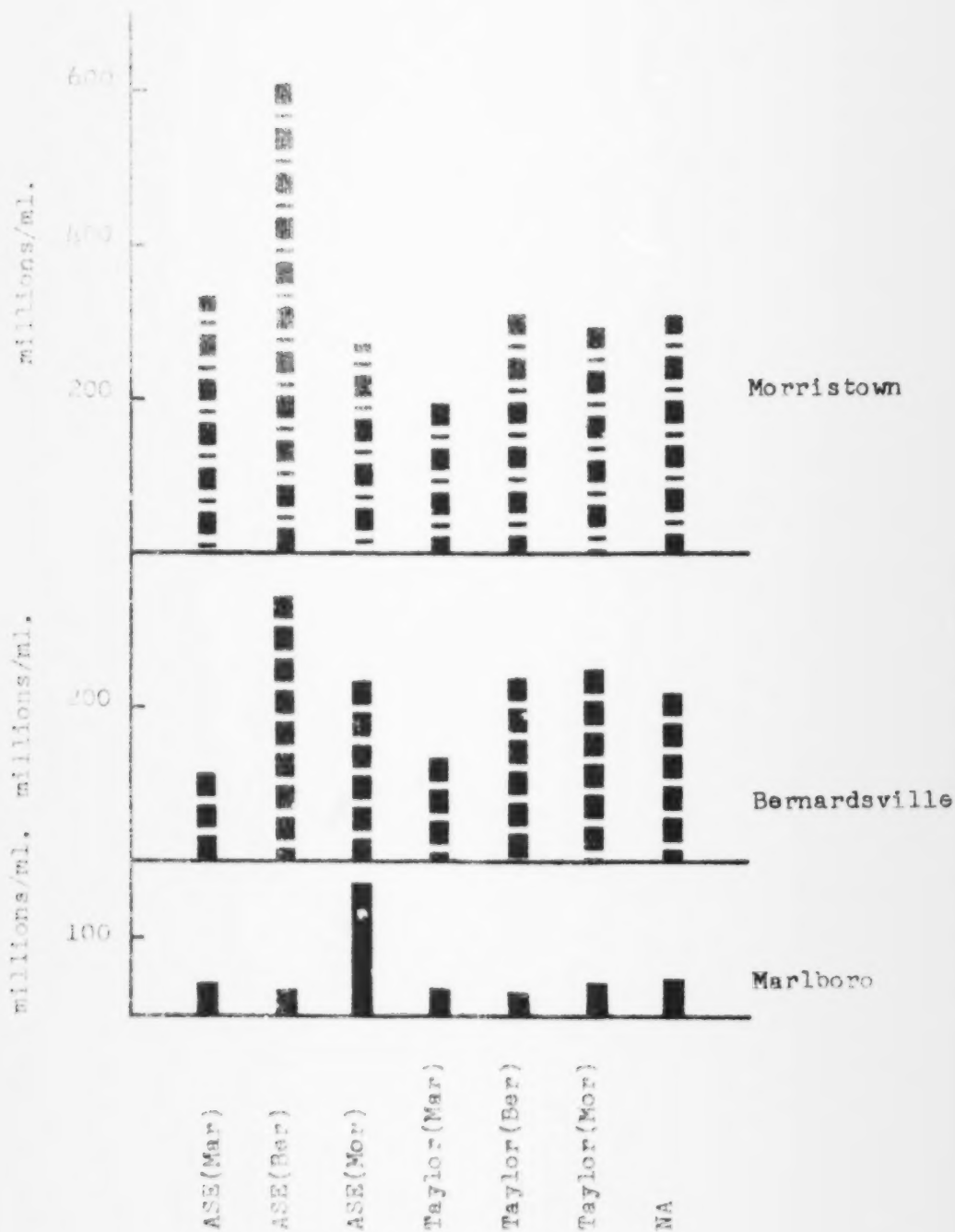


FIG. 2. VIABLE COUNTS OF SETTLED SEWAGE
ON DIFFERENT MEDIA

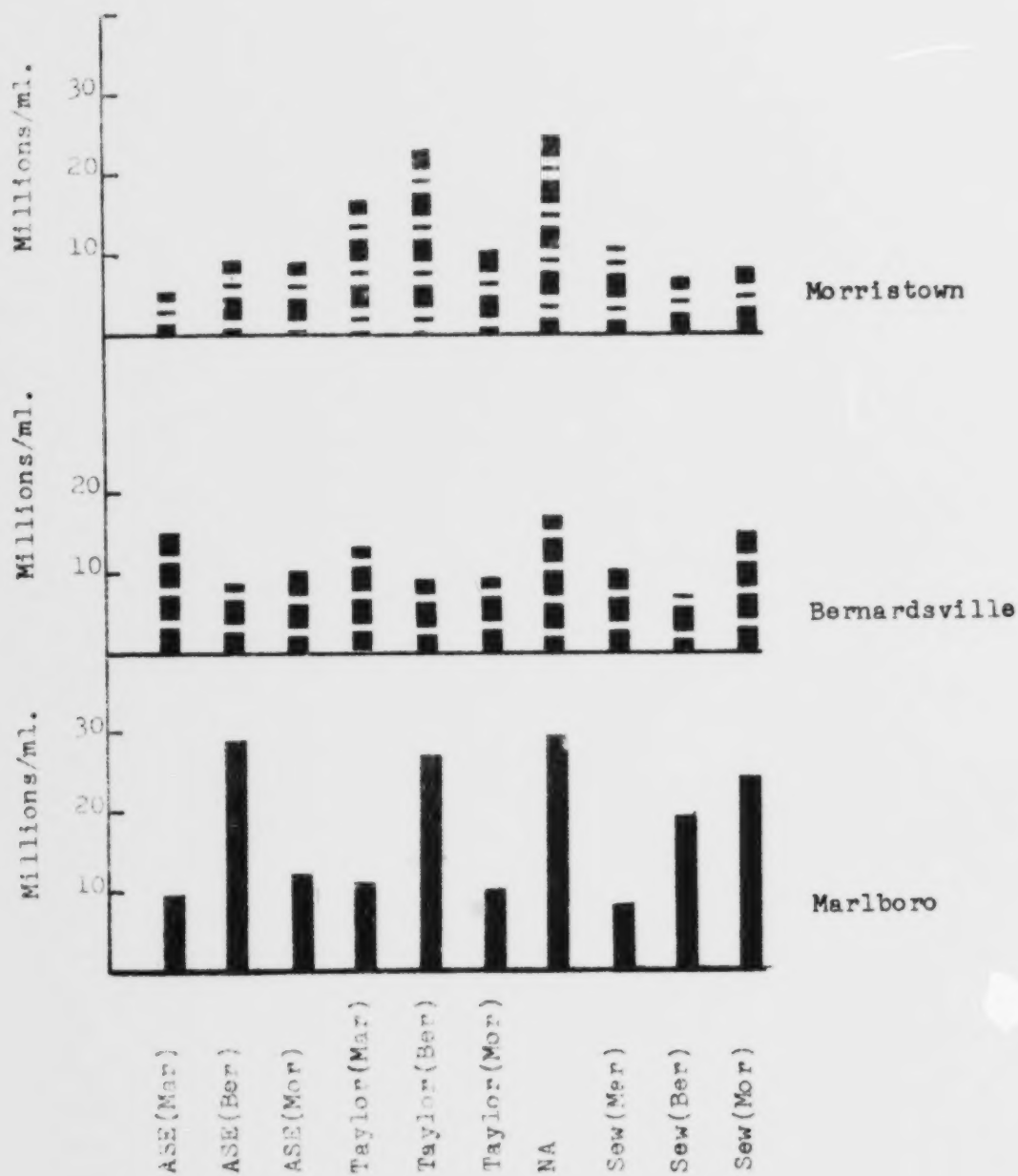


Table 7(a). Summary of Ranking of Viable Plate Count Media in Order of Effectiveness for Sewage by lsd. Plating Experiment VII.

Sewage.

Marlboro
Medium No.

8	1	6	4	3	10	9	5	7	2
8.88	97.3	9.99	10.63	11.76	13.47	19.27	28.41	29.27	29.27

Mean Count
(10^6 /ml)

Bernardsville
Medium No.

9	2	6	5	8	3	4	10	1	7
2.61	2.98	3.08	3.1	3.18	3.19	3.63	3.8	3.96	4.1

Mean Count
(10^6 /ml)

Morristown
Medium No.

9	10	3	2	6	8	1	4	5	7
7.08	8.07	8.94	9.43	10.11	11.09	15.52	16.81	22.94	24.8

Mean Count
(10^6 /ml)

Table 7 (b). Summary of Ranking Viable Plate Count Media in Order of Effectiveness for Activated Sludge by lsd. Plating Experiment VII.

Activated Sludge

Marlboro
Medium No.
Mean Count
(10⁶/ml)

5	2	4	6	1	7	3
23.04	29.16	31.36	37.21	39.69	43.56	114.5

Bernardsville
Medium No.
Mean Count
(10⁶/ml)

1	4	7	3	5	6	2
108.2	130	216.1	231	231	243.4	338.6

Morristown
Medium No.
Mean Count
(10⁶/ml)

4	3	6	7	5	1	2
196	265.7	295.8	309.8	309.8	331.2	610

The following numbers were designated to the media used in this experiment. (to be used with Figs. 1 and 2)

<u>No.</u>	<u>Medium</u>	<u>Code</u>
1	ASE (Marlboro)	ASE (Mar)
2	ASE (Bernardsville)	ASE (Ber)
3	ASE (Morristown)	ASE (Mor)
4	Taylor's medium (Marlboro)	Taylor (Mar)
5	Taylor's medium (Bernardsville)	Taylor (Ber)
6	Taylor's medium (Morristown)	Taylor (Mor)
7	Nutrient agar	NA
8	Sewage agar (Marlboro)	Sew (Mar)
9	Sewage agar (Bernardsville)	Sew (Ber)
10	Sewage agar (Morristown)	Sew (Mar)

As in other experiments, nutrient agar gave consistently higher counts for sewage. Other plating media gave counts of similar value on nutrient agar to a particular sewage sample, but did not rank in the same order of productivity for other sewages. Modification of Taylor's medium with Bernardsville activated sludge extract did not improve the productivity ranking above par with nutrient agar.

In these plating studies of activated sludge, ASE agars ranked as the most productive media. Marlboro activated sludge extract medium did not rank as the most productive medium as in previously reported experiments. Only Bernardsville activated sludge homologous plating gave maximum counts, whereas plating of Marlboro and Morristown sludges yielded maximum counts on heterologous ASE agars.

Discussion: The experiments described above showed that extracts of activated sludge (heterologous) yielded higher viable counts of activated sludge bacteria than any other medium tested. However, the ASE media varied in efficacy. In general, homologous plating may not necessarily yield higher counts; nevertheless, the counts obtained by homologous plating were not inferior to the counts obtained on nutrient

agar, a medium which was reported to have yielded maximum counts (Allen, 1944).

In the initial phase of the study, Marlboro ASE yielded the highest counts when activated sludges from Marlboro, Bernardsville and Morristown were plated on homo- and heterologous activated sludge extract media. However, this was not the case ten months later when a factorial experiment was conducted in which activated sludge samples from Marlboro, Bernardsville, and Morristown were plated at the same time on different media. In this study, the ASE media from Bernardsville and Morristown ranked as superior to Marlboro ASE medium, indicating that the productivity of these media changed as the result of fluctuation of some unknown variables. In the initial phase of this study, when Marlboro treatment plant was operating very satisfactorily, microscopic examination of activated sludge revealed high numbers of protozoa. Since these were found to be fewer at the time when the factorial experiment was conducted, it was suspected that plant operations had been altered. Inquiry revealed that important changes had been made in the operation of the treatment plant, suggesting that the mode of operation of the plant may have influenced the composition of the activated sludge, thus affecting the productivity of the extract medium.

Sewage agar was a poor medium for cultivation of aerobic heterotrophs of activated sludge. The sewage agars also varied in efficacy. Although some growth factors are present in sewage, it appears that metabolic activities of activated sludge bacteria produce factors conducive to elevating plate counts above those attainable with sewage. In the plating experiments, the soil extract medium was a poor substrate for obtaining high numbers of activated sludge bacteria, indicating that the factors contained in a soil extract medium were dissimilar to those of the most productive ASE and inadequate for supporting the growth of many activated sludge bacteria. It suggests, further, that the activated sludge flora is different from that of soil. A modification of Taylor's soil counting medium used in this study did not increase the productivity to a great degree. Taylor's and modified Taylor's medium yielded larger colonies than activated sludge extract media. A similar observation was made by Stevenson and Rouatt (1953) when soil bacteria were plated on original Taylor's medium.

With respect to media necessary for maximizing counts, sewage and activated sludge were distinctly different entities. Nutrient agar was a highly productive medium for the various sewages, although heterologous plating of these sewages on some extract media gave counts not significantly different from nutrient agar.

ADAPTABILITY OF THE REPLICA PLATE TECHNIQUE FOR CHARACTERIZATION
OF HETEROGENEOUS BACTERIAL POPULATIONS

A serious drawback in the differential enumeration of bacterial types is the difficulty or even the impossibility of precise correlation of numbers and properties of the bacteria obtained by plating separate samples from the same population on several selective media because of sampling errors in obtaining the inoculum and the lack of strict exclusiveness of the counts obtained on different kinds of media.

The replica plate technique devised by the Lederbergs (1952) for genetic studies seemed to be adaptable to improving the analysis of taxonomically or physiologically mixed populations since the differentiation of colonies can be done similarly to a great degree: i.e., by growth or other responses of the colonies imprinted from a master plate onto a series of culture media. This technique eliminates some sampling error and decreases the labor involved in characterizing the colonies by inoculating on a series of culture media. With this technique, the colonies growing on the surface of a master agar plate can be replicated on other agar plates by imprinting onto the surface of a piece of sterile velvet. Sterile agar plates of desired composition can be inoculated serially with identical inocula by impression against the velvet. Imprinted colonies of bacteria develop, according to their capabilities, in the same relative positions as on the master plate, to which the replica plate may be directly compared. Thus, information can be obtained relatively easily concerning practically every organism which grew on the master plate of the original sample. However, its application for characterizing quantitatively a very heterogeneous population like that of activated sludge requires validation because of certain inherent complications. There is the possibility that the texture of some colonies on the master plate may prevent transfer of the colonies. In contrast, some colonies may be depleted so that nothing of the colony is left on the master plate at some intermediate stage of the transfer series. Secondly, though the colonies may appear to be pure, some may be impure. Thirdly, interaction between the neighboring colonies through syntrophism or antagonism could give misleading nutritional results, elevated counts, or repression of colonies. These effects are often detected or eliminated if the organisms are isolated in pure culture. Fourthly, colony size affects the reproduction of colony form. Smearing of colonies may occur if the colonies are too large or if the piece of velveteen is unsuitable. This particular aspect is not a serious limitation because large colonies are seldom encountered on the ASE media used for the master plates. The effect of nutrient concentration on the colony size with a simple model is further discussed elsewhere.

The use of replica plating for the transfer of colonies of activated sludge bacteria which are distributed randomly on plates of non-selective media to plates containing various indicator media should permit a characterization of the population of aerobic heterotrophs, barring significant interference from the factors mentioned above.

To evaluate the influence of the above mentioned factors, preliminary experiments were performed using a simple artificial model system containing three bacterial strains as well as with the actual complex bacterial population of activated sludge.

Artificial Model: The bacteria used in the model studies consisted of Escherichia coli, Pseudomonas fluorescens, and an unidentified, red pigmented bacterium isolated from activated sludge. Experiments revealed that there was neither stimulation nor antagonism between these organisms.

For effective replica plating it is desirable to have small colonies on the plates, as mentioned previously. This can be accomplished by reducing either the incubation time or the nutrient concentration. With shorter incubation, some of the slow growers may not appear at all; hence the effect of nutrient concentration was also investigated. These investigations revealed that 1/25-strength casitone-glycerol-yeast autolysate (CGY) plating medium yielded small colonies of organisms in the model system. Individual streaking of colonies from master plates onto fresh CGY plates showed that more than 95% of the colonies were pure. The mixed nature of the remaining colonies was primarily due to merging of two adjacent colonies at their margins rather than simultaneous mixed growth. Thus, it can be concluded that the replica plate technique yielded highly reproducible results with the simple model described above.

Activated Sludge: The factors that probably limit the applicability of the replica plate technique for characterization of activated sludge bacteria were discussed above. Studies were conducted to investigate the degree of accuracy of this method taking into account all the aforementioned factors.

1. Failure of transfer of colonies grown on activated sludge extract plates.

To evaluate the effects of failure of transfer, two master plates were replicated; each on a set of five plates (ASE agar) and incubated at 28 C. After ten days, the replicated plates were compared with the corresponding master plates. The results are given in Table 8.

Table 8. Replication of Colonies Grown on Activated Sludge Extract Agar.

Replication No.	Master Plate I Number of Colonies = 176		Master Plate II Number of Colonies = 73	
	No. of Colonies Replicated	Percent of 176 Replicated	No. of Colonies Replicated	% of 73 Replicated
1	168	95.5	72	98.7
2	167	95.2	71	97.4
3	165	93.9	70	96.0
4	163	92.8	70	96.0
5	160	91.0	70	96.0
	<u>823</u>		<u>353</u>	

$$\% \text{ Weighted average} = \frac{823}{880} \times 100 = 93.7$$

$$\text{Variance}_1 = 3.41$$

$$\% \text{ Weighted average} = \frac{353}{365} \times 100 = 96.8$$

$$\text{Variance}_2 = 1.47$$

$$\bar{X} \text{ (grand mean)\%} = \frac{93.7 + 96.8}{2} = 95.2$$

$$s_p \text{ (pooled std. dev.)} = \sqrt{\frac{4(3.41) + 4(1.47)}{5+5-2}} = 1.25$$

(Z at 95% level, single tail test = 1.645)

$$\text{Then, the limit is: } 95.2 - 1.645 \times 1.25 = 93.1$$

Statistical analysis of the data in Table 8 indicated that a minimum of 93.1 percent replication of the colonies on the master plate can be accomplished at 0.05 significance level.

2. Estimate of error due to mixed colonies.

All the colonies from three ASE agar master plates were streaked on ASE agar to test their purity. Table 9 represents the results obtained.

Table 9. Mixed Colonies on Activated Sludge Extract Agar Master Plates.

Plate No.	Total No. of Colonies	No. of mixed colonies (observed)	Percent mixed in total
1	47	3	6.4
2	33	3	9.0
3	149	13	8.7

The percent weighted average of mixed colonies

$$\frac{19}{229} \times 100 = 8.3$$

The confidence limit for the number of mixed colonies can be computed as follows:

$$\begin{aligned} \text{C.L.} &= \text{Avg. percent of mixed colonies} \pm 1.96 \times \sqrt{\frac{p(1-p)}{n}} \\ &= 8.3 \pm 3.5\% \text{ at } 0.05 \text{ significant level.} \end{aligned}$$

where $p = 8.3/100$ and $n = 229$

The above data indicated that the probability of getting mixed colonies, which was between 4.8% and 11.8%, was within tolerable limits.

3. Validity of application of replica plate technique for nutritional characterization of activated sludge bacteria.

Colonies on an activated sludge master plate (ASE agar) were identified by numbers etched with a metal stylus on the bottom of the plastic petri dish. All of the colonies on the plate were picked with a needle, suspended separately in about 1 ml of sterile distilled

water. By means of replica plating the colonies on the same master plate were impressed on the following media of Lochhead's nutritional series in the order given:

1. basal medium
2. basal medium + yeast extract
3. basal medium + yeast extract + B₁₂
4. basal medium + amino acids
5. basal medium + amino acids + growth factors

(The composition of these media are appended to this report)

A loopful each of the prepared suspensions of cells was streaked on each of the above agar media. Also, all the plates, viz., the replica plates and the streaked plates, were then incubated at 28 C for 10 days and scores were given as + or - depending whether there was growth or no growth respectively. In this way isolates were obtained, purified, and separately tested. Each colony was also tested by replication imprinting. A statistical comparison of the replica plate method and streak plate method should indicate, within fiduciary limits, whether the replica plate method is as valid a method for characterization of heterogeneous population as the method of isolation and testing pure cultures. For this purpose the growth responses of all of the 127 colonies obtained by the replica plate method on the five Lochhead media were compared with the growth responses of the purified isolates on the same media. Each colony and corresponding isolate from the master plate were matched for ability or inability to grow on the same media. When there was a difference between responses of the replicated colony and the pure culture, a discrepancy was recorded regardless of which member of the pair failed to grow. The following table (Table 10) shows the number of discrepancies i.e., the number of colonies that showed opposite responses (growth or no growth) for each medium.

It seems, from the above table, that the number of discrepancies rises during the initial steps of replication and stabilizes in the latter ones. To evaluate the precision of the replica plate technique in such a situation, it is desirable to set a fiduciary limit based upon the results obtained in the last step of the operation, which is more rigorous a test in that it takes into account the discrepancies produced by preceeding steps in the replication procedure.

The confidence limit from the above data can be computed as follows:

Percent matching: 92.3

Percent non-matching: 7.7

$$C.L = 92.3 - 1.96 \times \sqrt{\frac{0.077 \times 0.923}{127}} = 87.8\%$$

Table 10. Discrepancies in Replica Plating.

Medium (Lochhead) series)	Total Number of Colonies on Master Plate	Total Number Accounted For	Number of Dis- crepancies	% Matching Colonies
1	127	126	1	99.3
2	127	119	8	93.8
3	127	116	11	91.4
4	127	117	10	92.3
5	127	117	10	92.3

Thus, it can be said that the replica plate method is reliable to about 88 percent at 0.05 significant level. Discrepancies arising in this series also included discrepancies due to nutritionally mixed (or impure) colonies.

4. Nutritional Characterization of Activated Sludge Bacteria with the Replica Plate Technique.

The growth responses of the colonies transferred by replica plate technique onto the Lochhead's media in the previous experiment were compared with the growth responses of the same colonies subjected to streaking on plates containing the same nutritional media.

Table 11 represents a comparative evaluation of the nutritional characterization of activated sludge bacteria on Lochhead media as done by replica plating and streak plating.

The higher growth response by the streak plate method was not altogether unexpected. It was partly due to the failure of transfer of some colonies from the master plate to the replica plate. One significant observation made in this experiment was that a substantial proportion (about 42 percent) of the total population from ASE failed to grow on any of the media either imprinted or streaked in this nutritional characterization study. This suggests that there are autogenous factors in the activated sludge which are required for the

Table 11. A Comparative Evaluation of Nutritional Characterization of Activated Sludge Bacteria by Replication Versus Isolation.

Total number of colonies on Master Plate: 127

Number of colonies that did not grow on
all of the replica plates 53

Percent of colonies that did not
grow on all of the replica plates. 42

Medium	Replica Plate Method		Streak Plate Method	
	No. of Colonies Grown	Percent Grown	No. of Colonies Grown	Percent Grown
basal medium	49	38.5	50	39.3
basal medium + yeast extract	56	44.1	64	50.4
basal medium + yeast extract + B ₁₂	59	46.4	70	55.1
basal medium + amino acids	62	48.9	72	56.7
basal medium + amino acids + growth factors	62	48.9	72	56.7

growth of these bacteria. In a separate experiment, 97% of the activated sludge bacteria growing on an ASE agar plate grew when reinoculated onto ASE agar.

In another experiment, colonies grown from activated sludge inocula on activated sludge extract plates were picked at random and inoculated into the series of Lochhead broth media. Table 12 represents the results obtained.

Table 12. Nutritional Characterization of Activated Sludge Bacteria by Random Sampling of Colonies.

Total number of colonies picked from activated sludge extract plates:	82
Number of colonies failed to grow on all of the media	20
Percent failed to grow	24

<u>Medium</u>	<u>No. of colonies grown</u>	<u>Percent grown</u>
basal medium	13	15.9
basal medium + yeast extract	47	57.3
basal medium + yeast extract + vit. B ₁₂	52	63.4
basal medium + amino acids	43	52.4
basal medium + amino acids + growth factors	55	67.1

Although the method of picking colonies at random was more biased than analysing the whole population, 24 percent of the colonies failed to grow in all the broth media indicating that some additional nutrients were needed besides the one employed in this investigation for the growth of activated sludge bacteria.

MICROBIAL ADAPTATION IN ACTIVATED SLUDGE

The term adaptation (synonym, acclimation), as commonly applied to activated sludge is loosely used to denote the adjustments made by the microbial community in response to changes in the environment. The recognized adaptive mechanisms include mutation, enzyme adaptation, modification of cell structures (e.g., spores, cysts, etc.) and changes in the composition of the population through death or shifts in predominance. Mutation must be supposed to be a constant occurrence, enzyme suppression has been reported by Gaudy (1962) and enzyme induction by McKinney, Tomlinson and Wilcox (1956) in artificial activated sludges.

It may be assumed that, when the tolerance of the cellular mechanisms is exceeded by environmental stresses, the population as a whole responds by the elimination of members least able to compete under the new conditions and by the multiplication of the most favored. There has been but little investigation in the area of the response of the aerobic heterotrophs along these lines.

One of the assumptions often made in projecting laboratory data to treatment plant operation is that an activated sludge developed on 'synthetic sewage' from sewage seed harbors a population analogous to the activated sludge of a sewage treatment plant. Further, it is presumed that the population responds to external stimuli in a fashion similar to that of the mixed flora of activated sludge, although such a response may not be possible if the population of the artificial system is not as varied as that of activated sludge. To investigate the nature of aerobic heterotrophic bacterial populations, studies were initially directed toward comparisons of the populations developed in laboratory activated sludge units on synthetic sewages in contrast with those of activated sludges from treatment plants.

Two experimental systems devised and studied earlier by McKinney, et al and Gaudy, et al, were investigated to study the aforementioned aspects.

Behavior of an Activated Sludge Developed on a Synthetic Waste Toward Aromatic Compounds.

Activated sludge was developed in the laboratory on a synthetic waste (meat extract, peptone, glucose and salts) by the fill-and-draw technique as described by McKinney, et al, ('56) in eleven cylinders. In about three weeks, a readily settleable sludge was formed. Samples from eight of the eleven sludges were plated on synthetic waste agar medium containing the nutrients and salts in the same proportion as in the synthetic sewage plus 0.85 percent Ionagar No. 2 added for solidification. Table 13 indicates the viable counts of the sludge grown in the laboratory units.

Table 13. Viable Counts of the Synthetic Waste Activated Sludge Units Before Adaptation.

Unit No.	Mean Counts on Synthetic Waste Agar (millions/ml)
1	335
2	313
3	286
4	301
8	354
9	316
10	340
11	291

Statistical analysis of the data indicated that there were no significant differences in the numbers of bacteria between sludge units and it may be reasonably assumed that the systems were very similar before adaptation. In studying the metabolism of aromatic compounds by an unacclimated heterotrophic population of a sludge grown on a synthetic medium, an estimate of the relative number of organisms having the physiological and genetic capability of metabolizing a specific substrate is of value. It is also of interest to investigate whether an analogous population distribution occurs in the activated sludge from a treatment plant. In the present investigation homogenized samples of laboratory-grown sludge and activated sludge (mixed liquor from an aeration tank) from a treatment plant were plated on synthetic waste agar, ASE agar and other plating media containing aromatics. The compositions of the synthetic waste agar and the plating media containing the aromatic compounds are given in the appendix. The aromatic compounds used in the plating media were: sodium benzoate, mandelate, phenyl acetate, p-hydroxy benzoate, anthranilic acid, benzyl alcohol, o-, m-, p- cresols, catechol, and phenol.

Table 14 represents the viable counts on different agar media of a sludge grown on a synthetic waste and of a treatment plant activated sludge.

Table 14. Bacterial Counts of a Laboratory-Grown Activated Sludge and Marlboro Treatment Plant Activated Sludge on Various Enumeration Media.

No.	Viable Count Medium	Laboratory Grown Activated Sludge Mean Count (10^6 /ml)	Treatment Plant Activated Sludge Mean Count (10^6 /ml)
1	benzoate	287	9.8
2	mandelate	299	38
3	phenylacetate	271	16
4	p-hydroxybenzoate	444	29
5	anthranilic acid	—	10
6	benzyl alcohol	186	4
7	o-cresol	18	0.035
8	m-cresol	45	0.01
9	p-cresol	134	0.08
10	catechol	0.0092	0.006
11	phenol	297	16
12	synthetic waste agar	233	146
13	ASE agar (Marlboro)	253	126

There were differences between the sludges in the number of organisms that were able to survive or utilize the various aromatics in the plating media. The viable counts for laboratory-grown activated sludge on any given medium were higher than the corresponding count for activated sludge.

The following conclusions can be drawn from the above data for the laboratory-grown activated sludge: Statistical analysis showed that the productivity of the synthetic waste agar and ASE agar (Marlboro) was not significantly different.

In ranking the productivity of the media by lsd test, there was no significant difference in the productivity of p-hydroxybenzoate, mandelate, phenol, benzoate, and phenyl acetate agar media. lsd test also indicated that there was no significant difference in the counts obtained on mandelate, phenol, benzoate, phenyl acetate, synthetic waste agar, activated sludge extract (Marlboro) agar, and benzyl alcohol agar.

The productivity of o-cresol, m-cresol, and p-cresol agars was comparatively poor. This was expected because of the toxicity of cresols. However, it was interesting to note that there was a considerable number of organisms in the laboratory-grown activated sludge, that could utilize the cresols.

Catechol plating medium was the least productive of all. Though considerable solids were built up, it was surprising to note that none of the plating experiments gave counts on 1×10^2 dilution. Plating at 1×10^3 dilution yielded colonies which were exclusively fungi.

Statistical analysis of the data on treatment plant activated sludge indicated the following:

There was no significant difference between the productivity of activated sludge extract agar and synthetic waste agar. These were the two media that gave the highest counts for activated sludge of Marlboro treatment plant.

All aromatic plating media supported only low numbers of activated sludge bacteria. However, there was a significant difference in their productivity. Of the aromatic plating media used in this study, mandelate, and p-hydroxy benzoate yielded the highest counts. Phenyl acetate, phenol, benzoate, and anthranilic acid media gave counts that were not significantly different from each other. Benzyl alcohol, p-cresol, o-cresol, m-cresol, and catechol yielded the lowest counts, there being no significant difference between the last four media.

Metabolism of Aromatic Compounds by Marlboro Activated Sludge and an Unacclimated Sludge Developed on a Synthetic Waste.

To investigate whether the activated sludge developed in a synthetic waste using Marlboro sewage as the seed had similar physiological activity in metabolizing aromatic compounds as the Marlboro treatment plant activated sludge, oxygen uptake studies with the Warburg respirometer were undertaken. An analysis of trends in the oxygen uptake of the resting cell suspensions would indicate whether the populations of the two sludge systems have the same constitutive enzyme systems or exhibit the same adaptive patterns in metabolizing the various aromatic substrates tested. It is not possible to include all the Warburg respirometer data in this report. The following conclusions were drawn from the oxygen uptake studies carried out with Marlboro treatment plant activated sludge and laboratory-grown activated sludge.

A distinct increase in the oxygen uptake, though with a long lag, by the Marlboro activated sludge was observed with benzoate, mandelate, phenylacetate, p-hydroxy benzoate, and benzyl alcohol. There was no appreciable oxygen uptake with anthranilic acid, o-, m-, p- cresols, catechol, and phenol. This absence of uptake might have been the result of either toxicity or of unavailability of the substrate to the microbial population.

The laboratory activated sludge exhibited metabolism, though with appreciable lag periods, with most of the aromatic compounds tested except with o- and m-cresol. The lag period was shorter in the case of the laboratory activated sludge than that of Marlboro sludge. It can be concluded from these studies that the potential of the unacclimated laboratory-grown sludge for metabolizing the various aromatic compounds was higher than that of Marlboro sludge, although it was difficult to accomplish quick degradation of the aromatic compounds by the unacclimated sludge prior to adaptation.

Behaviour of the Sludges Acclimated to Aromatic Compounds.

It is clear from the preceding studies that the laboratory-grown activated sludge, even though unacclimated to aromatics, and the Marlboro treatment plant activated sludge are physiologically different entities with respect to the ability of oxidizing aromatics. Furthermore, the distribution of microorganisms able to use a specific aromatic compound was quantitatively different. To study the effect of the process of acclimation on occurrence, nature, and behaviour of microorganisms in the acclimated sludges, 11 sludge units were adapted during four weeks to the following aromatic compounds: benzoate, mandelate, phenylacetate, p-hydroxybenzoate, anthranilic acid, benzyl alcohol, o-cresol, m-cresol, p-cresol, catechol and phenol. The acclimation by gradual

addition of aromatics and simultaneous withdrawal of glucose and nutrient broth from the synthetic waste feed was performed as described by McKinney, *et al.* A twelfth, unadapted laboratory-grown sludge, was designated as control sludge. Viable counts of these sludges were obtained by plating homogenized and serially diluted samples of an acclimated sludge on the homologous aromatic agar medium. The composition of these media are given in the appendix. The effect of prolonged feeding of aromatic compounds as the sole source of carbon to these sludges was further followed by noting the changes in the suspended solids and viable counts. Table 15 represents the viable counts and suspended solids of the various acclimated sludges.

Comparison of the results obtained for control sludge (refer to Table 14) and results obtained for sludges acclimated for a long time (Table 15, column 6) indicated the following:

In many cases there was a significant difference between the viable counts of the control sludge on a specific aromatic plate and the viable counts of the corresponding acclimated sludge plated on a homologous medium.

Though control sludge (Table 14, column 3) gave high counts on benzoate, the same sludge during acclimation to benzoate gave fewer counts on benzoate (Column 3 of Tables 14 & 15). The same trend was noted in the case of mandelate, p-hydroxybenzoate, benzyl alcohol, phenol and p-cresol. In contrast, the sludge acclimated to phenylacetate gave higher counts than the counts obtained for control sludge on phenyl acetate plates.

In this study, no direct relationship was found between suspended solids and viable counts. Though prolonged feeding of aromatics showed a definite increase in the suspended solids and viable counts, the viable counts did not increase in proportion with the suspended solids (columns 3 and 4 vs. columns 6 and 7 in Table 15). In sludge units acclimated to phenylacetate ~~by prolonged feeding~~ a reduction in viable counts occurred due to prolonged feeding of aromatics (columns 3 and 6 in Table 15). However, in the remaining units there was an increase in the bacterial numbers.

Viable Counts on Sludge Extract Media.

Though the viable counts were relatively high for most of the acclimated sludges on homologous plating media, no colonies grew when the sludge acclimated to catechol was plated on catechol agar medium. There was, however, an increase in the mixed liquor suspended solids. Microscopic examination of the acclimated sludge used for inoculation revealed large numbers of fungal hyphae and bacteria; therefore, the absence of colonies on catechol agar plates was not purely due to toxicity. To test whether some sorts of microbial associations existed in these sludge columns, i.e., whether the products of metabolism of

Table 15. Changes in the Viable Counts and Suspended Solids Due to Prolonged Feeding of Aromatics to Sludges.

Sludge acclimated to	Acclimated sludge plated on agar medium containing	Viable counts on the day the sludge units started receiving only aromatics as sole source of carbon Mean (10^6 /ml)	Suspended solids mg/l	No. of days aromatics fed	Viable counts after prolonging of aromatics Mean (10^6 /ml)	Suspended solids
benzoate	benzoate	39	3900	14	60	8730
mandelate	mandelate	107	3460	21	448	4990
phenylacetate	phenylacetate	645	3790	15	299	7300
p-OH benzoate	p-OH benzoate	165	3360	42	393	4970
anthranilic acid	anthranilic acid	97	3540	27	162	5760
benzyl alc.	benzyl alc.	66	3900	15	153	3890
o-cresol	o-cresol	29	2600	28	147	5800
m-cresol	m-cresol	29	3430	29	75	5610
p-cresol	p-cresol	42	4540	26	—	—
catechol	catechol	0	3740	33	0.013	6310
phenol	phenol	47	3560	23	381	5070

one species influenced the growth of another, inocula of acclimated sludges were plated on extracts of homologous sludges. The sludge extract plates were made by autoclaving about 250 ml of the sludge specifically well-acclimated, filtering the settleable solids, and adding about 0.85% Ion agar No. 2. Sludge extract plates containing, in addition, nutrient broth and glucose were also prepared and the viable counts of the acclimated sludges were obtained by homologous plating to study whether nutrient broth and glucose caused any change in the viable counts.

Table 16 represents the viable counts of acclimated sludges on the corresponding sludge extract plates and on other media.

Table 16. Viable Counts of Sludges Adapted to Aromatic Compounds on Extract and Synthetic Waste Agars.

Sludge Acclimated to:	Sludge Extract + Nutrient broth + Glucose		
	Sludge Extract mean (10^6 /ml)	Nutrient broth + Glucose mean (10^6 /ml)	Synthetic Waste Agar mean (10^6 /ml)
benzoate	652	382	230
mandelate	53	68	62
phenylacetate	83	108	101
p-hydroxybenzoate	123	72	43
anthranilic acid	6	7	3
benzyl alcohol	106	105	73
o-cresol	188	215	55
m-cresol	228	314	156
p-cresol	76	76	29
catechol	192	268	39
phenol	172	165	60

A comparison of the above results with the viable counts of sludges subjected to prolonged feeding of aromatic compounds (Refer Table 15) indicates that, in general, there are two factors involved in the magnitude of viable counts. Firstly, there seems to be an aromatic substrate dependency. Lower bacterial numbers were obtained when there was no external supply of mandelate, phenyl acetate, *p*-hydroxy benzoate, anthranilic acid, and phenol in the plating media. In contrast, in the case of benzoate, *o*-cresol, *m*-cresol, *p*-cresol, and catechol and counts were much higher on the sludge extract plates. Probably, the increase was not due simply to exclusion of a toxic aromatic substrate from the plating medium since plating of acclimated sludge samples on synthetic agar (salts + nutrient broth + glucose) gave fewer counts than counts on sludge extract plates. Presumably, the great majority of bacteria in catechol and other sludge units were mutually dependent on metabolites or other physical and chemical factors that were produced by the microorganisms themselves (autogenous nutrients). These growth promoting substances presumably were readily available in an extract made from the sludge itself rather than originating from homologous plating medium. If catechol were completely toxic to the microorganisms in the mixed culture, there would not have been any microbial activity after prolonged feeding of catechol as the only source of carbon and as a result no growth could have resulted even on catechol sludge extract plates. Hence, it was quite probable that the microbial activity in the above organic milieu was maintained by some associative relationships amongst microorganisms. The pattern of growth in the different situations points to accessory growth factors as the most likely beneficial factors rather than energy sources or assimilative materials.

Metabolism of Aromatic Compounds by Sludges Acclimated to Aromatic Compounds. The oxygen uptake of washed cells (suspended in M/20 phosphate buffer) of the 11 sludges acclimated to various aromatic compounds was measured with homologous as well as with heterologous aromatic substrates in the main compartment of the Warburg flasks. Since it is not possible to present all the Warburg respirometer data, a summary of the results is presented in Table 17 based on the type of lag obtained for various substrates with a given aromatic acclimated sludge.

The respirometer data indicate that the acclimated sludges had the ability to metabolize compounds which are neither closely related nor fit in the metabolic pathway of the substrates to which the sludge was acclimated. This suggests that the sequential induction pattern of Stanier either does not operate in heterogeneous systems like activated sludge or that if it does heterogeneous populations such as used in these experiments do not exhibit the patterns.

Table 17. O₂ Uptake Patterns of Various Acclimated Sludges.

Sludge acclimated to	Substrates Tested		
	No lag with significant O ₂ uptake	Lag but significant O ₂ uptake	Prolonged lag and low O ₂ uptake
benzoate	benzoate, catechol	mandelate, p-hydroxy benz, benzyl alc, o-, p-cresols, phenol	anthranilic acid <u>m</u> -cresol
mandelate	benzoate, mandelate catechol	phenylacetate anthranilic ac benzylalcohol, o-, <u>m</u> -, <u>p</u> -cresols phenol	p-hydroxy benzoate
phenylacetate	mandelate, phenyl- acetate, <u>p</u> -OH benz. catechol	benzoate, anth- ranilic acid, benzylalcohol phenol	<u>o</u> -, <u>m</u> -, <u>p</u> - cresols
<u>p</u> -hydroxy benzoate	<u>p</u> -OH benzoate, catechol	mandelate, anthranilic acid, benzylalcohol, <u>m</u> -cresol, phenol	benzoate, phenyl- acetate, <u>o</u> - cresol
anthranilic acid	anthranilic acid, catechol, <u>p</u> -OH benz.	mandelate, phenyl acetate, benzylalc. <u>o</u> -, <u>m</u> -, <u>p</u> -cresols, phenol	
benzyl alcohol	benzoate, catechol, anthranilic acid, <u>p</u> -OH benzoate, benzyl alcohol	phenyl acetate <u>o</u> -, <u>m</u> -, <u>p</u> - cresols	mandelate
<u>o</u> -cresol	benzoate, anthranilic acid, catechol, phenol and <u>o</u> -cresol	<u>p</u> -OH benzoate, benzylalcohol phenylacetate, <u>m</u> -, <u>p</u> -cresols mandelate	

Table 17. (Continued)

Sludge acclimated to	Substrates Tested		
	No lag with significant O ₂ uptake	Lag but significant O ₂ uptake	Prolonged lag and low O ₂ uptake
<u>m</u> -cresol	benzoate, <u>m</u> -, <u>p</u> -cresols, catechol, phenol	mandelate, phenyl acetate, <u>p</u> -OH benz. anthranilic acid, benzyl alcohol, <u>o</u> -cresol	
<u>p</u> -cresol	benzoate, <u>p</u> -OH benz. benzyl alcohol, <u>p</u> -cresol, catechol	mandelate, phenyl- acetate, anthranilic acid; <u>o</u> -, <u>m</u> -cresols phenol	
catechol	benzoate, catechol, anthranilic acid	mandelate, phenyl- acetate, benzylalc. <u>o</u> -, <u>m</u> -, <u>p</u> -cresols phenol	
phenol	benzoate, anthranilic acid, catechol, phenol	mandelate, phenyl- acetate, <u>p</u> -OH benz. benzylalcohol, <u>o</u> -, <u>m</u> -, <u>p</u> -cresols	

Population Characterization Studies. The differences in physical appearance and in the microscopic characteristics of the acclimated sludges were strong evidence, though inconclusive, that there was a population shift during the process of adaptation of the laboratory-grown activated sludge to the aromatic compounds. In the current investigation, an effort was made to characterize the population harbored by all the acclimated sludges on the basis of their ability to utilize aromatic compounds. The replica plate technique was used to determine the number of organisms capable of growing on homologous and heterologous plating media. A sample of an acclimated sludge was homogenized, serially diluted and plated on the homologous medium (salts + nutrient broth + glucose + aromatic compound). Catechol sludge extract medium was used for plating catechol-acclimated sludge because the synthetic waste agar containing catechol did not support growth of catechol sludge bacteria. These plates were used as master plates. By means of the replica plate technique the colonies grown on master plates were inoculated on heterologous plating media. The master plates and the replica plates were incubated for about seven days at 28 C and scores were given as + or - for each colony depending on whether there was growth or absence of growth on different media. Colonies utilizing a particular set of substrates were placed in one category and the number of such colonies in the category was reported as 'frequency'. Each group of colonies utilizing substrates of a specific combination was designated by a 'Type code No.' which can be considered as analogous to a species or strain designation. Code numbers were given to the types arrayed in order of increasing numbers of substrates utilized, so that, in general, lower code numbers represent types using the larger numbers of substrates. Table 18 lists the type code numbers assigned to colonies based on their ability to utilize specific combinations of substrates.

Figures 3, 4, 5, and 6 indicate the general types and their frequency of occurrence in the sludges acclimated to various aromatic compounds.

This study indicated that the structure of the acclimated sludges as exhibited by the different types of organisms harbored were quite different. In general, there were population shifts in the sense that the preponderance of the types was not the same in the various sludges acclimated to different aromatic compounds. It appears from the data that environmental selection of types was very severe in certain cases, as with sludges acclimated to o- and m-cresols, yet the surviving types were versatile with respect to substrate utilization.

Table 18. Code Numbers Assigned to Colonies According to their Growth on Different Plating Media Containing Aromatic Compounds.

<u>Substrates Utilized</u>	<u>Type Code No.</u>
1-11	1
1-9, 11	2
1-8, 10-11	3
1-7, 9-11	4
1-6, 8-11	5
1-9	6
1-8, 11	7
1-7, 9, 11	8
1-7, 10-11	9
1-7, 9-10	10
1-6, 8-9, 11	11
1-6, 8, 10-11	12
1-6, 9-11	13
1-5, 7-9, 11	14
1-4, 6-9, 11	15
1-2, 4-9, 11	16
1-8	17
1-7, 11	18
1-7, 10	19
1-6, 8-9	20
1-6, 9, 11	21

Table 18. (Continued)

<u>Substrate Utilized</u>	<u>Type Code No.</u>
1-6, 9-10	22
1-6, 10-11	23
1-4, 6-8, 11	24
1-4, 6, 9-11	25
1-7	26
1-6, 11	27
1-6, 10	28
1-6, 9	29
1-4, 6-8	30
1-4, 6-7, 11	31
1-4, 6, 9, 11	32
1-6	33
1-4, 6-7	34
1-4, 6, 11	35
1-2, 4-6, 11	36
1-2, 6-7, 10-11	37
2-4, 6-8	38
2-4, 6-7, 11	39
1-4, 6	40
1-2, 4-6	41
1-2, 6-7, 11	42
2-4, 6, 11	43
1-4	44
1-3, 6	45
1-2, 4, 6	46
2-4, 6	47
1-2, 6	48

FIG. 3 FREQUENCY OF THE GENERAL TYPES OF BACTERIA
IN SLUDGES ACCLIMATED TO AROMATIC COMPOUNDS

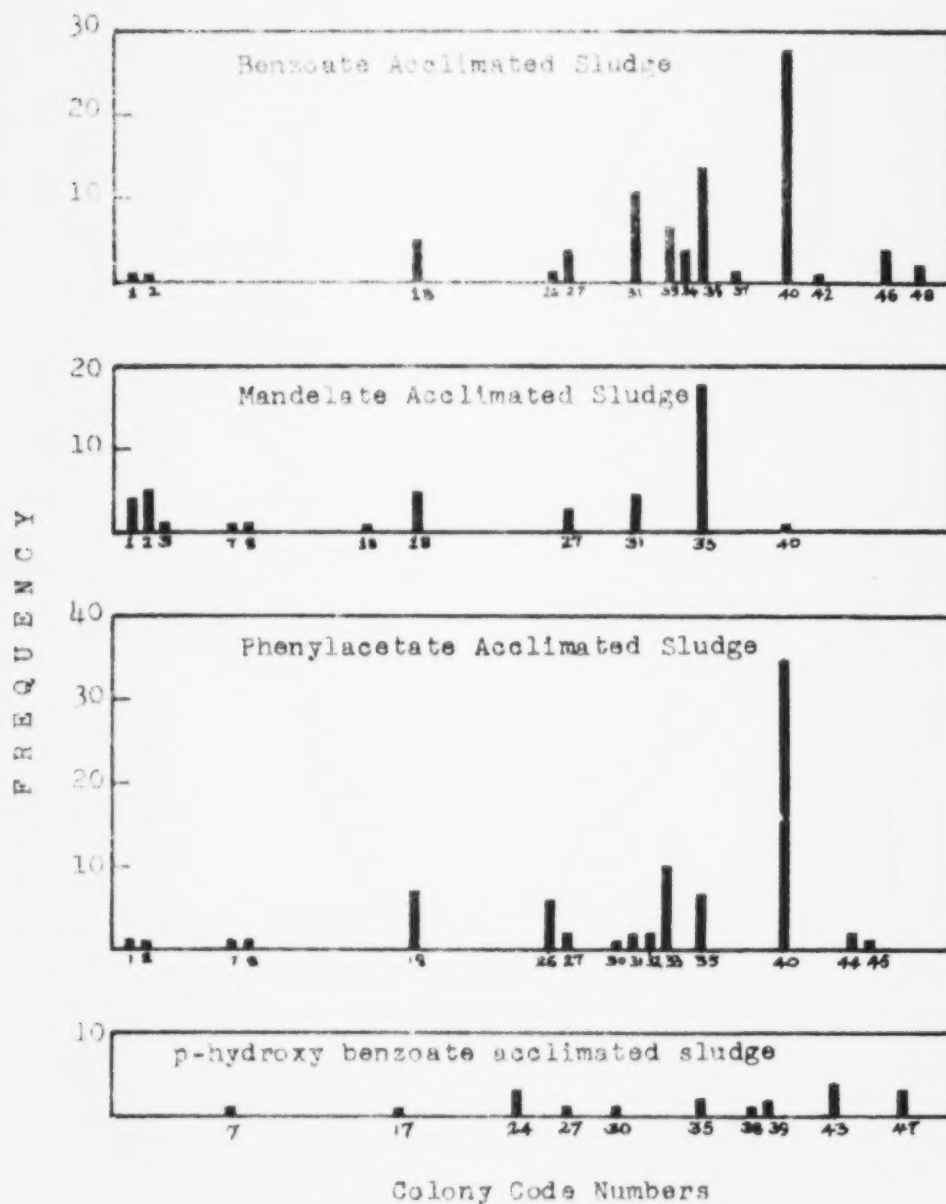


FIG. 4. FREQUENCY OF THE GENERAL TYPES OF BACTERIA
IN SLUDGES ACCLIMATED TO AROMATIC COMPOUNDS

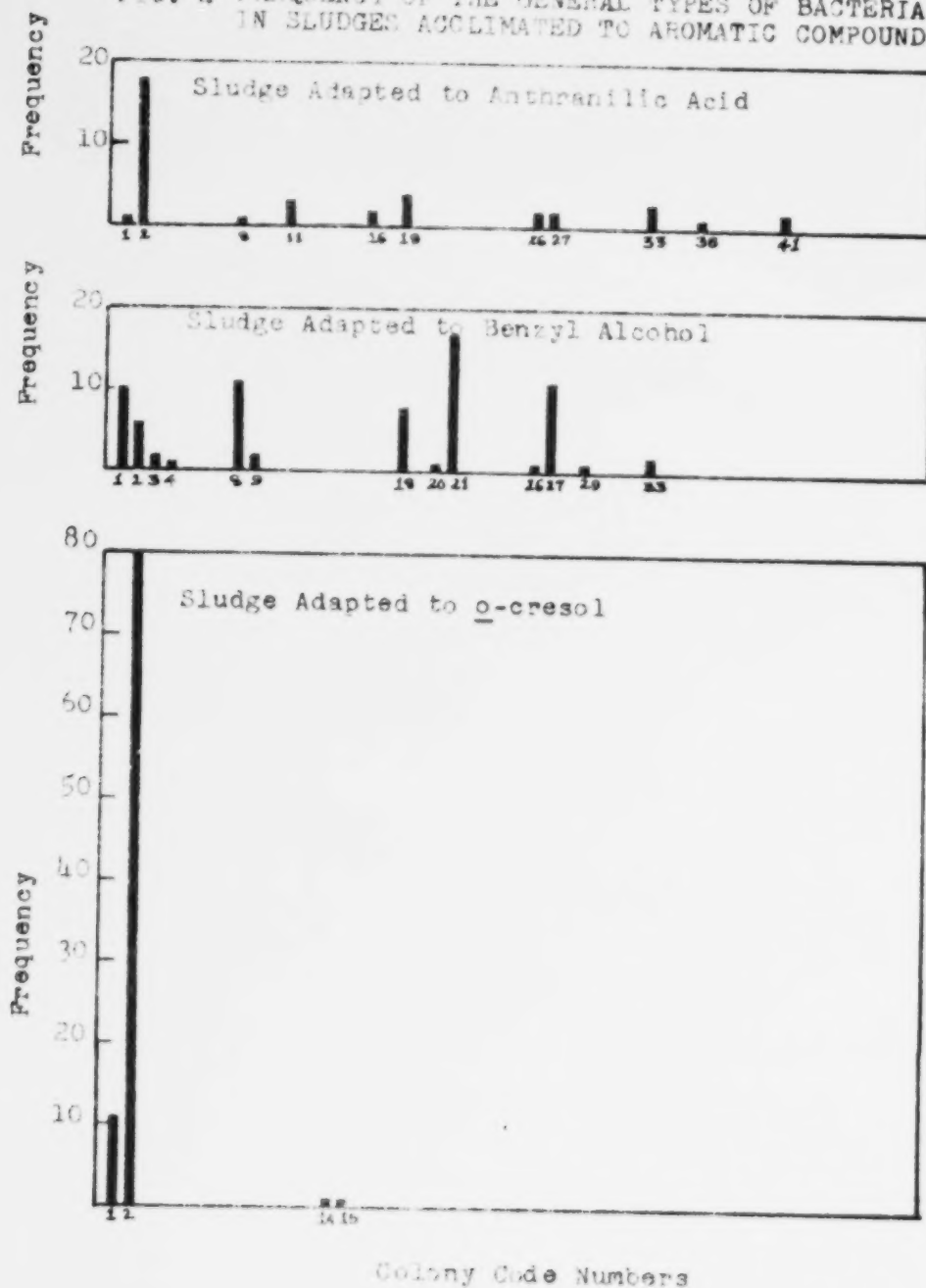


FIG. 5 FREQUENCY OF THE GENERAL TYPES OF BACTERIA
IN SLUDGES ACCLIMATED TO AROMATIC COMPOUNDS

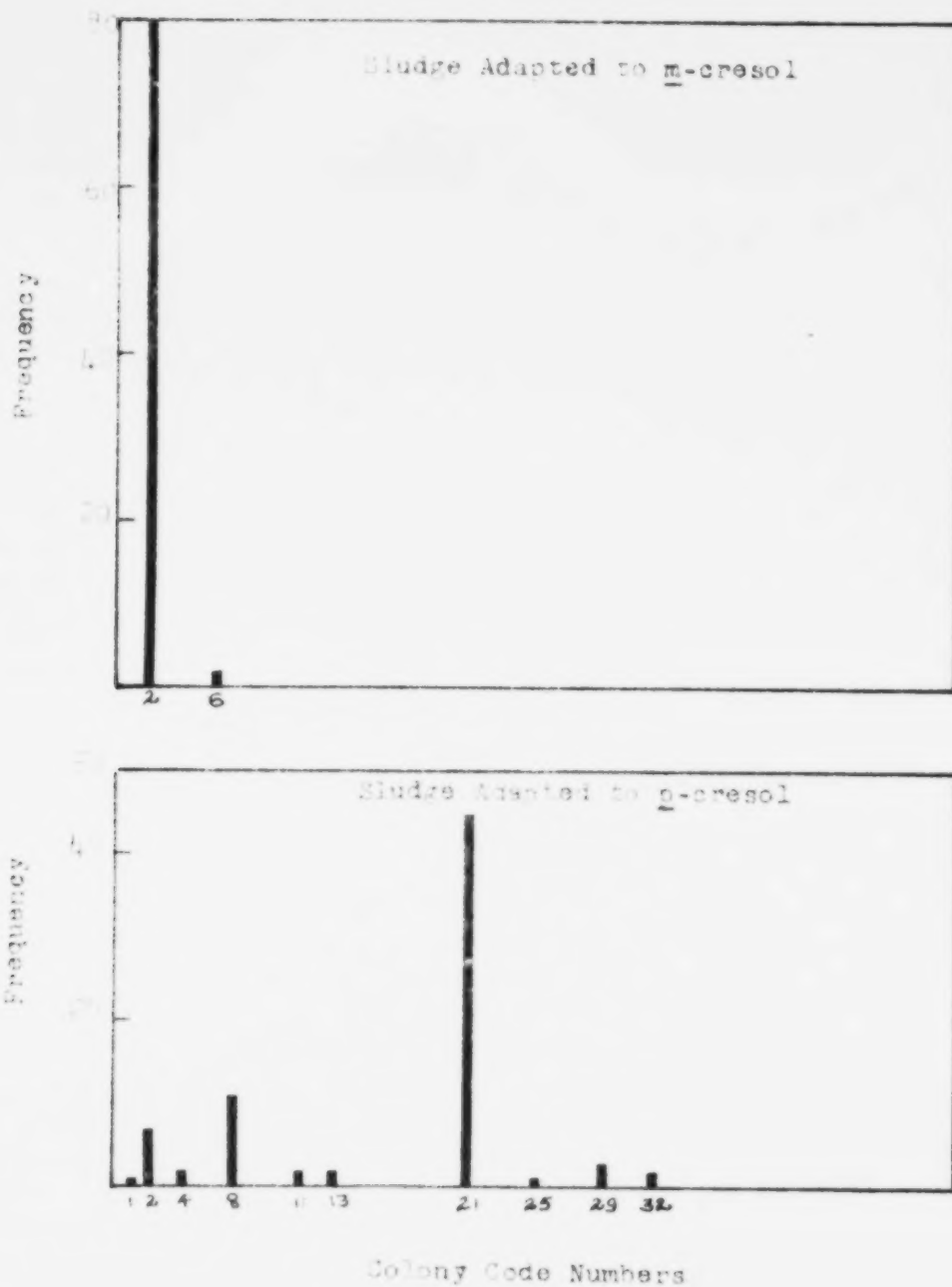
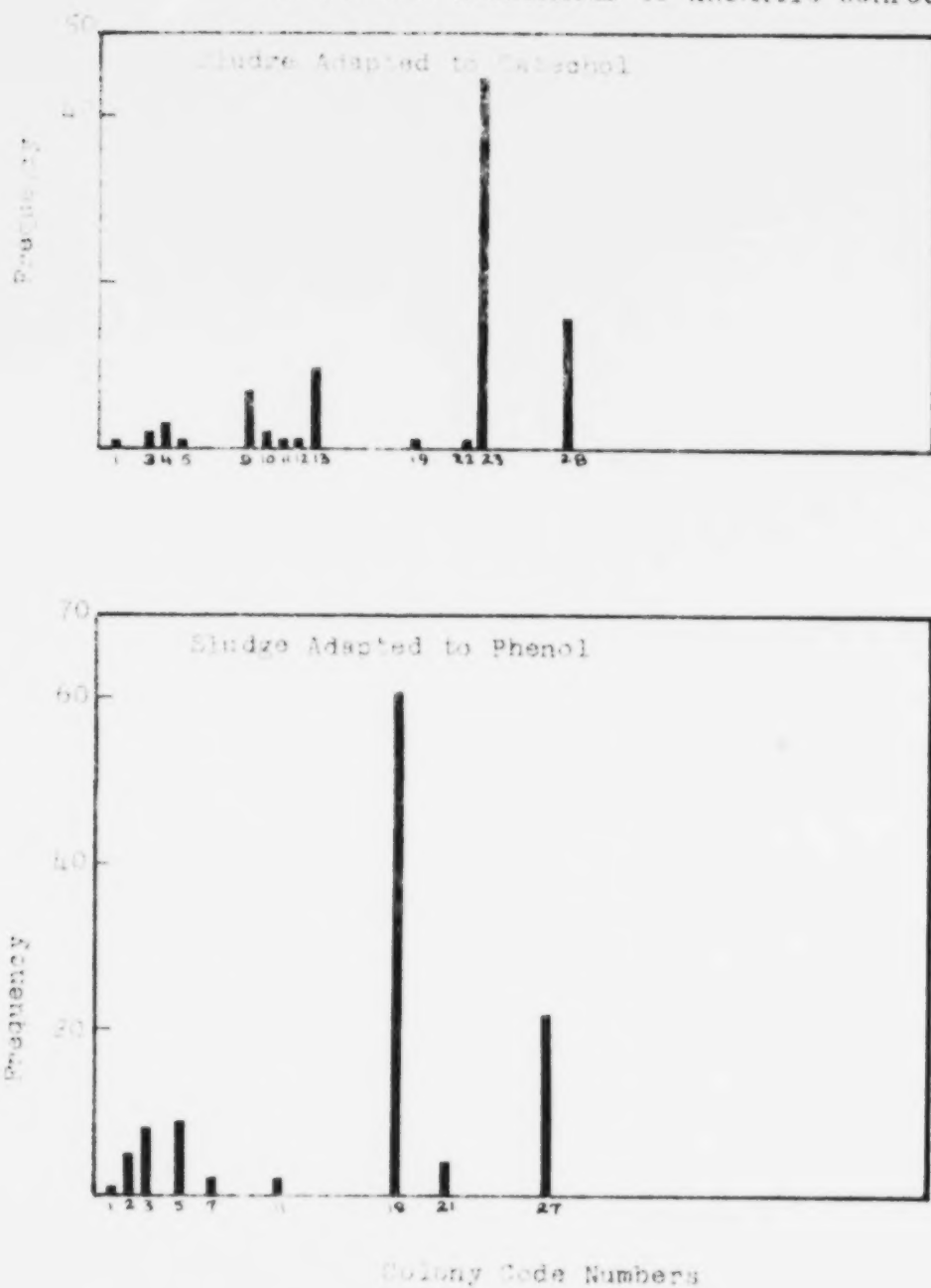


FIG. 5 FREQUENCY OF THE GENERAL TYPES OF BACTERIA
IN SLUDGES ACCLIMATED TO AROMATIC COMPOUNDS



Discussion. In the present investigation it was found that the activated sludge developed by the fill-and-draw systems using Marlboro sewage as the seed was radically different from the Marlboro treatment plant activated sludge, with respect to the distribution of bacteria having the ability to utilize the selected group of aromatic compounds.

The metabolic activity, as measured by oxygen consumption, of the activated sludges developed on a synthetic waste was higher than that of a treatment plant activated sludge. In the metabolism of aromatic compounds, the activated sludge developed on a culture medium containing nutrient broth and glucose exhibited comparatively shorter lags in metabolizing a greater number of compounds tested than Marlboro plant activated sludge. This readiness may have been due to acclimation of the organisms by some aromatic compounds present in nutrient broth. It was interesting to note that catechol, a key intermediate in the pathway of benzoate and mandelate metabolism, was not oxidized by Marlboro activated sludge, although benzoate and mandelate were oxidized.

A comparison of the viable counts on various plating media containing aromatic compounds indicated that laboratory-grown activated sludge inocula yielded significantly higher counts than Marlboro activated sludge on most of the plating media. Presumably the bacteria associated with these higher viable counts were responsible for degradation of a greater number of aromatic compounds.

In these investigations, the fill-and-draw systems of activated sludge could be acclimated to 750 mg/l of aromatic compounds. Physical and microscopic examination indicated differences in the physical characteristics of acclimated sludges as well as in the biota. Sludge acclimated to o-cresol and phenol were, respectively, orange and golden-yellow but catechol sludge was black and contained many hyphae of fungi. Sludges acclimated to benzoate, mandelate, p-hydroxybenzoate, phenylacetate, anthranilic acid, benzyl alcohol, m-cresol, and p-cresol were light yellow to brown. The population characterization study indicated that each sludge harbored specific types which had the ability to use a particular group of compounds. At present there is not sufficient evidence to say that such types are characteristic of a given sludge. There was a difference in the selection of types by the acclimated sludges depending on the compound to which they were acclimated. Thus o- and m-cresols contained very few types while other acclimated sludges harbored several types. However, the oxidative activity of these few cresol types encompassed those of the several types in other populations.

The concepts of simultaneous adaptation developed by Stainer did not seem to be applicable in terms of the lag phase patterns of enzyme induction to the mixed populations. Compounds which were not metabolically related were oxidized equally well by a mixed population. Benzoate was postulated as an intermediate in the breakdown of mandelate and it was shown that Pseudomonas fluorescens acclimated to benzoate

oxidized mandelate with a lag. In the present studies, it was observed that many of the acclimated sludges oxidized benzoate and mandelate equally and the benzoate acclimated sludge oxidized mandelate without a lag. The mixed population of the sludge acclimated to p-hydroxy benzoate oxidized catechol without any lag. This was decisively shown not to be the case with pure cultures (Sleeper 1950). The sludge acclimated to catechol was an exceptional system. Although benzoate was separated by many intermediates in the benzoate-catechol metabolic pathway, sludge acclimated to catechol oxidized by all of the acclimated sludges without a lag, no colonies grew when the acclimated sludges were plated on catechol agar. The failure to grow suggests that the populations harbored by most of the sludges acclimated to aromatic compounds could not assimilate the carbon of catechol but could oxidize it. The plating of catechol-acclimated sludge on catechol-sludge extract gave very high counts in comparison to those on catechol agar, suggesting that there exists an association in the bacterial community of the sludge and that the microbes depend on the intermediate products of catechol metabolism or on the metabolites produced by the variety of organisms present in the sludge environment. The types of organisms present in activated sludge which supply essential substances to the activated sludge community and may not subsist on exogenous nutrients deserve investigation as to their relation to the other organisms.

The metabolic activity of sludges acclimated to o- and m-cresols was not the same, though there was no significant difference in the metabolic potentiality as judged by the ability of the predominant population (type No. 2) to grow on aromatic substrates. The sludges acclimated to m-cresol oxidized benzoate, catechol, m-cresol and phenol without a lag while the sludge acclimated to o-cresol oxidized benzoate, catechol, o-cresol, anthranilic acid and phenol without a lag. Similarly, though the percentage incidence of type No. 40 was approximately the same in sludges acclimated to benzoate and phenyl acetate, their metabolic activity was dissimilar. The interrelationships between a predominant population and a secondary population and repression by substrate interaction with the enzyme systems of the different species, probably play a role in dictating the metabolic pathways of aromatic compounds in mixed cultures.

The data presented in this investigation indicated that the validity of interpreting lag phases as evidence for simultaneous adaptation technique in predicting the pathway of metabolism of aromatic compounds by mixed cultures was unreliable.

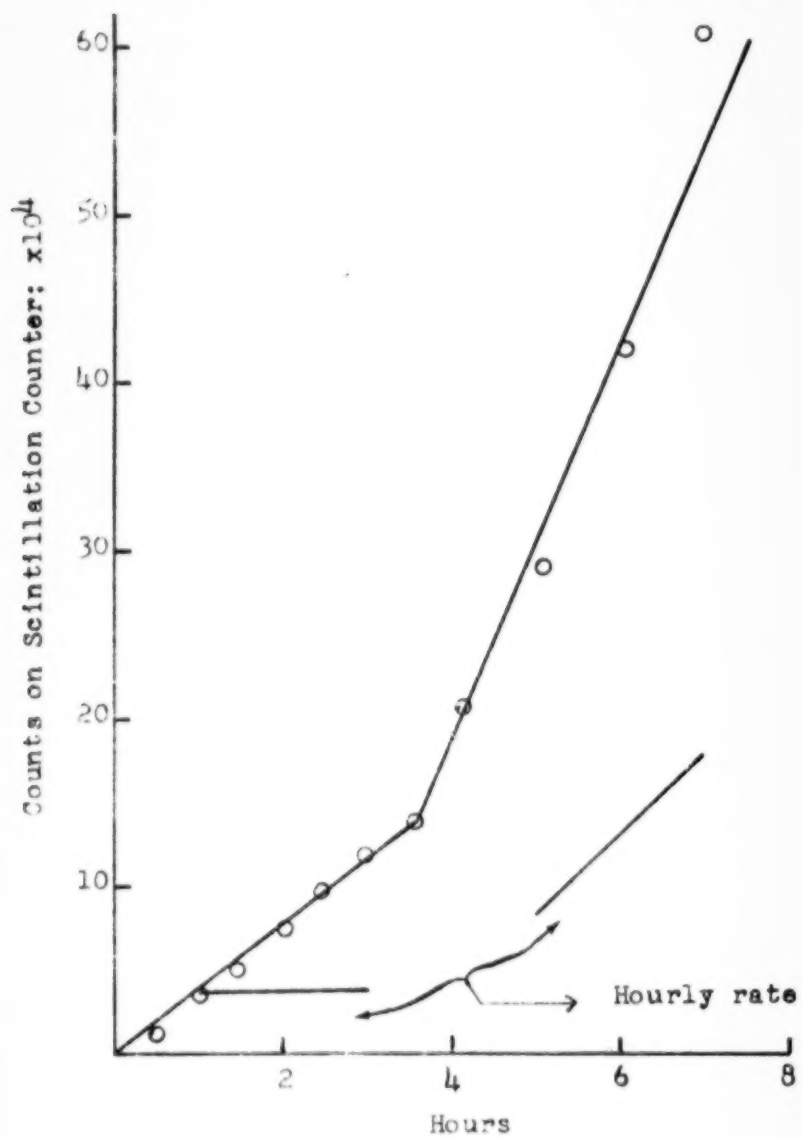
Studies on Induction and Repression. In a previous progress report, the results obtained on induction and repression experiments on a sorbitol-glucose medium employed by Gaudy (1962) were reported. Subsequently, further studies with radioactive sorbitol were carried out for specifically detecting the utilization of sorbitol in the presence of glucose.

Because of the possibility of accumulation of incompletely oxidized products in the cells or in the medium, an experiment was performed with sorbitol labelled in either the no. 1 or the no. 6 carbon and with a mixture of both. Twenty μ c of C^{14} -sorbitol were added to three reaction vessels containing 50 ml of washed cells of adapted sludge suspended in M/20 phosphate buffer. Then, unlabelled sorbitol and glucose were added to yield a resultant concentration of 300 mg/l of each. The concentration of adapted sludge solids was 110 mg/l. The utilization experiment was performed at room temperature (27 C). The evolved CO_2 was swept from the culture vessels, precipitated as $BaCO_3$ in the collecting tubes, and counted in a liquid scintillation counter. Collecting tubes were replaced by fresh tubes at 30 minute intervals.

Labelled CO_2 was evolved at equal rates regardless of the position of the C^{14} . The results obtained with the label in no. 1 position are presented in Fig. 7. It is clear that a constant, but relatively small, fraction of the sorbitol was attacked during the first 3.5 hours (approximately 4×10^4 counts/hr), following which the utilization of sorbitol was much more rapid (about 13×10^4 counts between the 5th and 6th hours). Numerous reproducible experiments measuring the utilization of glucose both chemically and manometrically in this system have shown that the glucose is largely consumed in about 4 hrs. These results were interpreted as proof that the utilization of sorbitol by the adapted sludge was very largely, although not completely, suppressed in the presence of glucose. The results correspond closely with those of Monod (1942, p. 180, 181) in experiments with Escherichia coli growing in a mixture of mannitol and xylose. The xylose was utilized at a low rate until mannitol was consumed, whereupon, the rate of xylose utilization increased.

Experiments with activated sludge from the treatment plant were performed manometrically and with radioactive sorbitol for comparison with the sorbitol adapted system. The details for the C^{14} -tracer experiments were similar to those of the experiments with adapted sludge experiments, except for the following modifications. Five microcuries of $1-C^{14}$ -sorbitol were used instead of 20 microcuries. The concentration of glucose and sorbitol in the mixed substrate was 150 mg/l of each, and sorbitol when used alone was 300 mg/l. The activated sludge MLSS were 245 mg/l.

FIG. 7 UTILIZATION OF SORBITOL BY ADAPTED SLUDGE



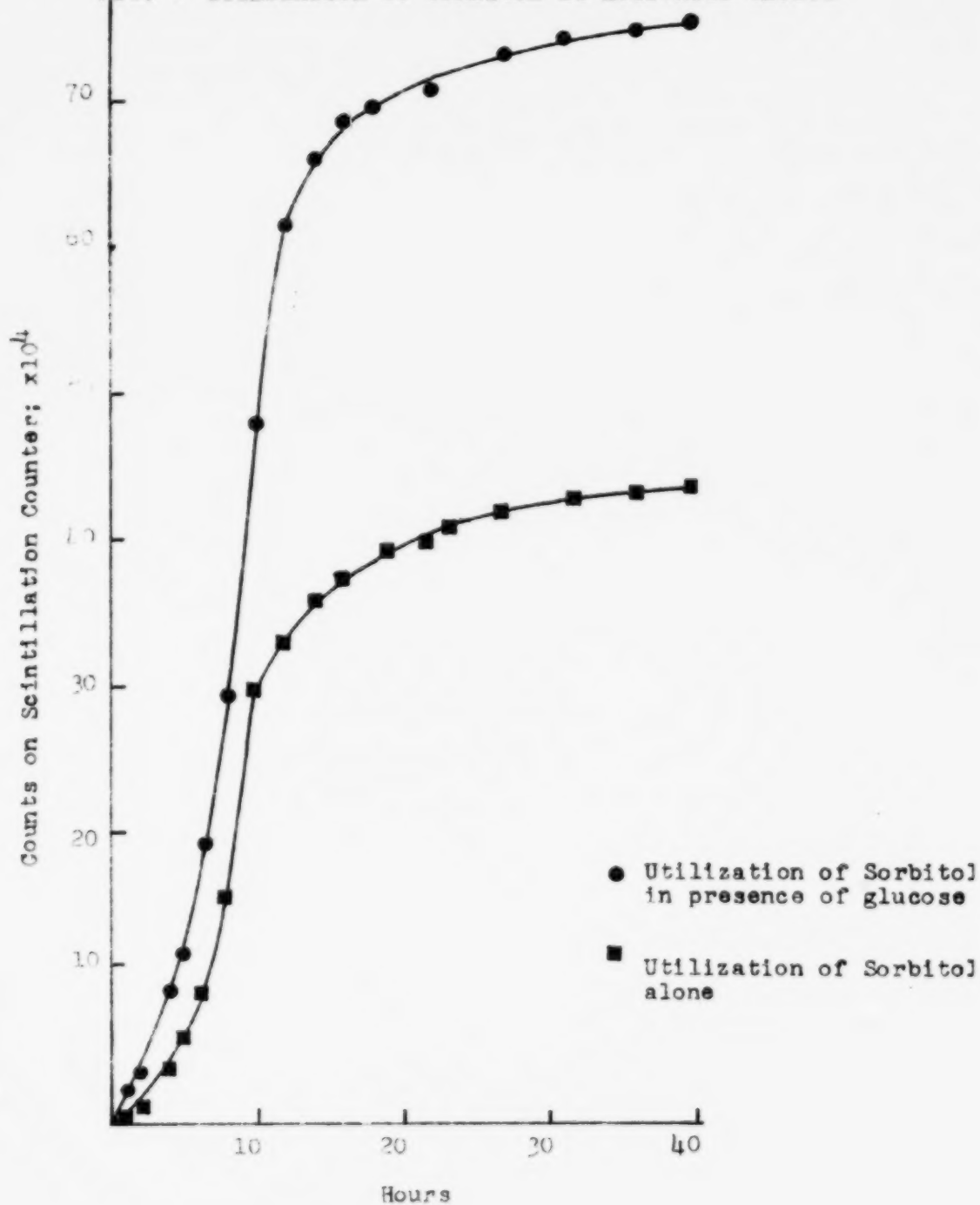
In the Warburg respirometer studies, both adapted and treatment plant activated sludge were used. Fifty ml. of mixed liquor, freshly collected from the aeration tank (2450 mg/l MLSS), was placed in 125 ml Warburg flasks, with alkali in the center well for oxygen consumption measurements. The adapted sludge was washed in M/20 phosphate buffer and resuspended in basal medium 1 to give 110 mg cell solids per liter. In one run with adapted sludge, 300 mg/l of each carbohydrate was used. In a second run with activated sludge only, 300 mg/l of each was used when taken individually, 150 mg/l when used together. Respirometer experiments were performed at 30 C.

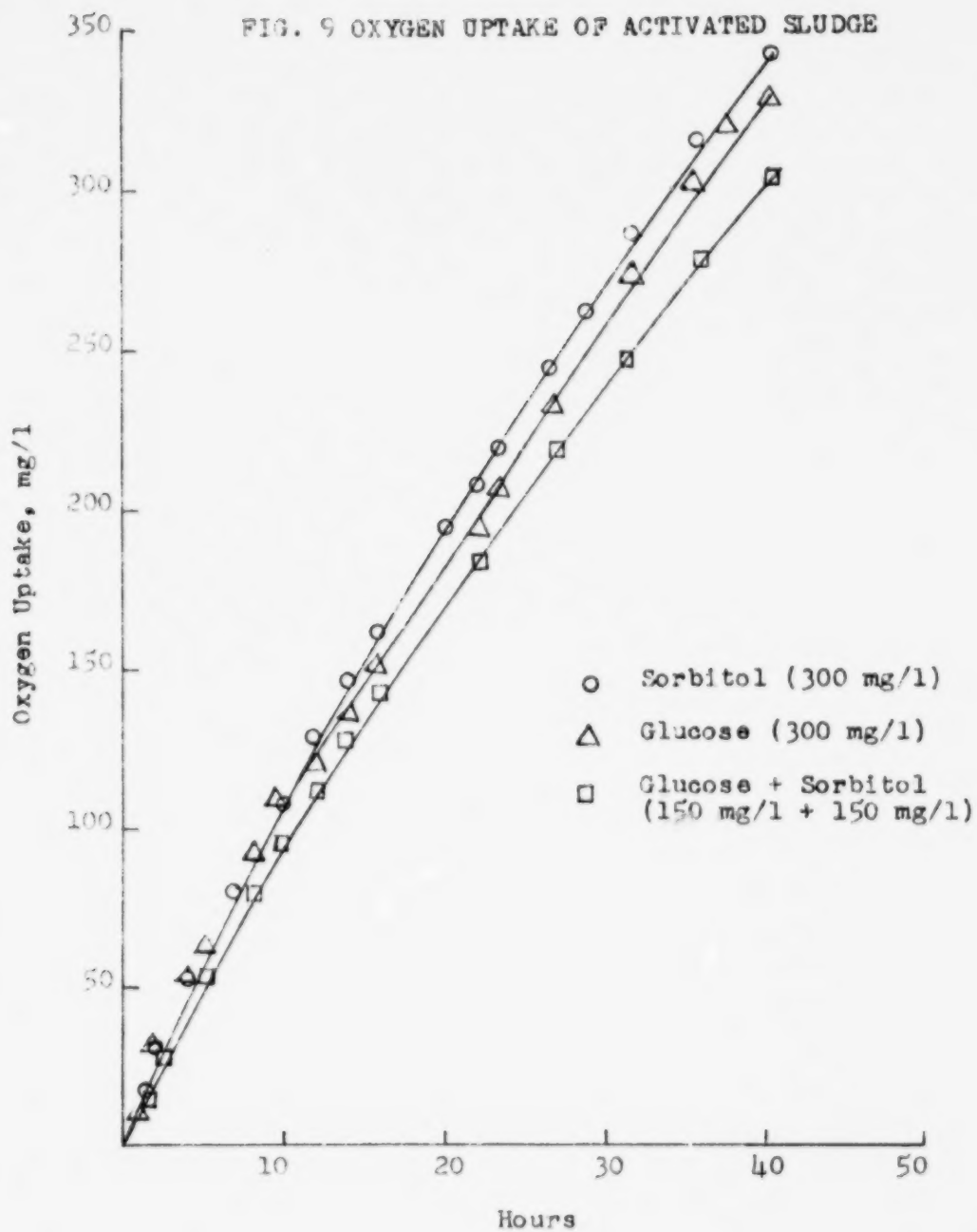
The evolution of $C^{14}O_2$ is shown in Fig. 8. In contrast to the suppression of sorbitol utilization by adapted sludge, the treatment plant activated sludge utilized the sorbitol in the presence of glucose as readily as in its absence. Since there was an initial lag period of about 4 to 5 hours in both curves, it cannot be attributed to repression by exogenous glucose. It should be noted that the lag in these experiments did not correspond to a suppression of sorbitol utilization by glucose. $C^{14}O_2$ was evolved at an accelerating rate from the beginning of the experiment. There was no corresponding lag in the oxygen uptake curves; however, the high rate of endogenous respiration of the activated sludge may have obliterated the lag in oxygen uptake due to exogenous substrates.

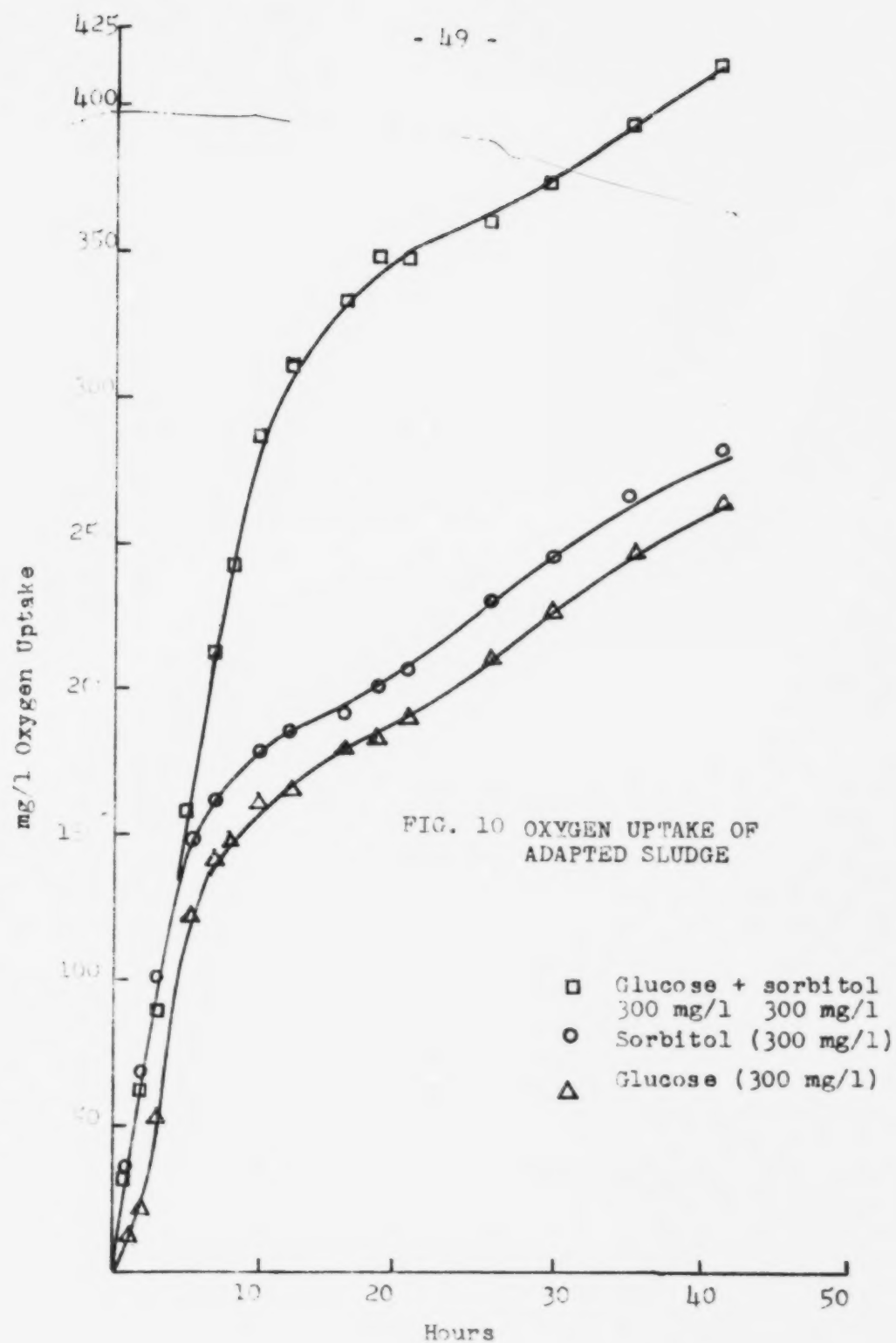
In spite of the rapid initial rates of sorbitol utilization by the activated sludge in both cases, the evolution of $C^{14}O_2$ from the reaction vessel containing sorbitol almost ceased at about 60% of that evolved from the vessel containing both glucose and sorbitol. We had insufficient information to hazard speculation on this phenomenon.

The conclusions to be drawn from the Warburg respirometer experiments (Figs. 9 and 10) are limited. The principal information sought was, however, available; namely, that the activated sludge was capable of using either and both carbohydrates without prior adaptation. Comparison of rates of oxygen uptake between activated and adapted sludges could not be fairly made because of the differences in cell solids concentration, those of the adapted sludge being much lower. Furthermore, there was no way of making a comparison of numbers of active organisms. Nevertheless, taking manometric and isotopic experiments together, it may be said that, although the adapted sludge showed the effects of repression by glucose, the activated sludge was able to attack both substrates immediately and simultaneously.

FIG. 8 UTILIZATION OF SORBITOL BY ACTIVATED SLUDGE







Bacteriological Studies. There was little morphological heterogeneity in the types of cells seen in the adapted sludge. The predominant bacteria were rods of varying length and slenderness, many showing slight curvature. There appeared to be three or four classes of cell sizes. Many cells were actively motile. Flocculation or aggregation of cells, beyond occasional microscopic clumps and incompletely separated cells undergoing division, was not evident. The adapted culture which developed in our experiments, although referred to as sludge, was a dense, well dispersed suspension of rod-shaped bacteria which would settle, however on standing quietly for a time.

Allen found that standard nutrient agar was a good medium for the enumeration of activated sludge bacteria because it yielded high counts in comparison with other culture media. The counts of the adapted sludge on nutrient agar showed few, if any, statistically meaningful differences from counts on the carbohydrate supplemented basal agars.

To determine whether obligate microaerophilic organisms formed sufficient proportion of the adapted sludge population to elevate the count, pour plate counts were compared with surface spread plate counts. Table 19 represents the counts obtained on the various substrates.

Table 19. Pour Plate Counts and Surface Plate Counts of Adapted Sludge on Different Substrates.

Substrate	Pour Plate Counts $10^6/\text{ml}$	Surface Plate Counts $10^6/\text{ml}$
Glucose	198	230
Sorbitol	179	250
Glucose + Sorbitol	171	257

Statistical analysis of the above results indicated that the surface plate counts were higher than the pour plate counts.

The search for bacteria incapable of forming colonies under plating conditions employed was continued by means of total cell counts performed with the Coulter electronic particle counter. The cell counts obtained with this instrument were unreliable because of the interference by particulate matter contained in the basal medium and also due to the inconstant numbers of the non-cellular particles during growth. However, Petroff-Hausser microscopic cell counts were higher than the viable plate counts by about two-fold in absolute values. The lower viable count was not probably due to the effect of the diluent used because there was no significant difference between the counts obtained by using distilled water or basal medium as diluents. This was regarded as evidence suggestive that the basal agars plus carbohydrates were not capable of supporting the maximum possible yield of colonies from the adapted population, i.e., that the colony counts were biased toward low values. Such a possibility was suspected from the impression of different size classes of cells in the adapted population. To investigate whether there were other types of bacteria capable of multiplying on autogenous nutrients formed in the sorbitol adaptation medium by the growth of the primary population, the 72-hours population of an acclimated sludge was plated on basal medium + sorbitol and also on plates of staled sorbitol medium.

The staled medium was prepared by sterilizing a portion of the 72 hrs. flask or sorbitol adaptation medium with its content of cells and growth products, and by adding 0.85% Ionagar No. 2 to the liquor obtained after filtration. The liquor with the agar was sterilized once more before pouring the plates.

Table 20 represents the results obtained with acclimated sludges developed by using several sewages as initial seed.

The above data showed no significant difference between the counts obtained on basal agar and staled medium plates indicating that, by statistical evaluation, there was no significantly large cryptic population.

Population Characterization. To estimate the number of types of bacteria in the adapted sludge, all colonies grown from the plates seeded for counting on basal agar and the staled medium plates were subjected to several differential tests. Coliforms were characterized by the confirmed test using brilliant green bile broth. Table 21 indicates the types of organisms found in the adapted sludge

Table 20. Viable Counts of Adapted Sludge on Sorbitol Medium and Staled Medium.

Adapted sludge developed from sewage of	Viable Counts On	
	Sorbitol medium (10 ⁷ /ml)	Staled medium (10 ⁷ /ml)
Marlboro	53.3	42.9
Donaldson Park	17.9	11.9
Marlboro	46	37.3

characterized on the basis of differential tests used in this investigation. It can be seen from Table 21 that there were only a few types of organisms in the adapted sludge, unlike the flora of the activated sludge of a treatment plant. Further, the data was suggestive that an enrichment of type 1 species, coliforms and type 4 species had occurred during acclimation, since these accounted for 75% of the population.

Inocula of the isolates obtained in the above experiments from both staled medium and sorbitol medium were transferred to two set of tubes containing basal medium with sorbitol and sorbitol plus yeast extract to determine whether yeast extract had a stimulatory effect on the growth of the isolates. All the tubes containing the yeast extract yielded luxuriant growth after 24 hrs. while 32% and 40% of the total isolates from the staled medium plates and sorbitol agar plates respectively did not exhibit an equivalent growth in the tubes lacking yeast extract, although microscopic examination after incubation for four days showed viable bacteria. The substantial growth of the yeast-stimulated colonies on both kind of plates viz., the ones containing the basal medium plus sorbitol and the ones containing staled medium, may have been due to the presence of some stimulatory nutrients in the agar used in the preparation of the plates or to syntrophism between neighbouring colonies. These two factors did not operate when individual colonies were transferred to broth cultures, so that the stimulation by the yeast may be regarded as evidence for a tendency to syntrophism.

Table 21. Differential Reactions of the Bacteria Present in Sorbitol
Adapted Sludge.

Type No.	Gram stain	Gelatin	Dex.	Lac.	Mal.	Sor.	Sta.	Suc.	Organisms isolated on		
									Sorbitol Agar	Staled Medium	% of total isolates
									% of total isolates	% of total isolates	% of total isolates
1	(1)	-	-	-	-	-	-	-	40	43.9	
2	-	-	A	-	-	-	-	-	16.7	7.37	
3	-	-	A	-	A	A	-	A	3.3	4.87	
4	-	-	A	A	A	A	A	A	6.6	24.39	
5	Coliforms (2)	-							33.3	17.07	
6	-	+	A	-	A	-	A	-	-	2.4	

- (1) A. Acid production.
- No reaction.
(2) Reactions at 30 C.

Comparison of growth on sorbitol agar and on staled medium shows a different distribution of types. The counts on the staled medium probably represent a true picture of the population. Although statistical analysis of the counts indicated that the total viable counts were not significantly different (i.e., difference may exist, but not proven by counts), the fact that isolates were benefited by yeast extract, which can be regarded as analogous to bacterial extract in staled medium, indicated again that a mixed population of bacteria builds up nutrients autogenously and bacteria in the population become dependent upon these additional nutrients for growth either with or without the exogenous nutrients supplied in the medium.

Discussion.

From the evidence presented as to the restricted composition of the adapted population, it may be concluded that such a population is largely confined to the adaptation processes associated with physiologically uniform cultures. The most immediate effective reaction consists of revising the enzyme economy of the cells in response to the nutrient substrate. In a population in which all of the cells are subject to enzyme repression, the result is to delay the utilization of some available substrate. In a truly mixed population, whatever enzyme repression takes place appears to affect only a part of the population, leaving the remainder free to attack the available substrates immediately.

In the model glucose-sorbitol system reported upon here, the process of cellular adaptation was confirmed; however, the concomitant loss of ability to utilize the double substrate was not due to the repression of enzymes in a heterogeneous population, but in a physiologically homogeneous one.

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57. A

A P P E N D I X

Statistical Analysis of the Plate Count Data.

The computations involved in the statistical analysis of plate count data is illustrated below by an example using small integers for simplicity. The statistical analysis of the results of a typical plating experiment (plating experiment VII) is also included in this appendix.

Example: The following simple data is examined to determine whether there is any significant difference between the means of each set of observations.

Data on Plate Counts:

	V I A B L E P L A T E C O U N T S		
	Medium I	Medium II	Medium III
	1	4	9
	4	9	16
	<u>9</u>	<u>16</u>	<u>25</u>
Mean, $\bar{x}$	4.33	9.66	16.66

Since the above counts follow the Poisson distribution, analysis of variance can not be done directly unless the data is transformed to approximate the normal distribution. Square root transformation is one of the transformations which can be used to normalize a set of data that follows the Poisson distribution. This transformation involves taking the square root of each of the observations in the data.

Subjecting the individual plate counts to square root transformation we have:

Transformed Data:

	Medium I	Medium II	Medium III
	$\sqrt{x}$	$\sqrt{x}$	$\sqrt{x}$
	1	2	3
	2	3	4
	<u>3</u>	<u>4</u>	<u>5</u>
Mean of transformed data = $\sqrt{\bar{x}}$	2	3	4

The mean count for each medium can be computed by squaring $\bar{x}$. Thus,

$$\text{Mean count on medium I} = 2^2 = 4$$

$$\text{Mean count on medium II} = 3^2 = 9$$

$$\text{Mean count on medium III} = 4^2 = 16$$

Note that these means are slightly different from the arithmetic means obtained for the original data. (The mean counts given throughout this report were derived from the transformed data.)

Analysis of variance was performed on the transformed data according to Dixon and Massey (1957).

a) Analysis of Variance (transformed data).

		df	mss	
Total sum of squares	93	9		
Correction factor	81	1		
Total corrected sum of squares	12	8		
Within media	6	2	3	$F = 3/1 = 3$
Residual	6	6	1	

$$\text{Tabulated } F_{(2,6 \text{ df})} = 5.99$$

Hence, we can hypothesize that there is no significant difference between the means of the three media at 0.05 significant level.

b) Testing the Differences in Means Using lsd (least significant difference).

$$\text{lsd}_{(0.05)} = t_{(0.05)} \times s_{\bar{d}}, \text{ where}$$

$$t_{(0.05)} = \text{tabular value for error degrees of freedom}$$

$$s_{\bar{d}} = 2 \sqrt{s^2/r}, \text{ with } s^2 \text{ the error variance and}$$

$$r = \text{number of observations per mean}$$

For the differences between two means to be significant at the 5% level the observed differences must exceed the lsd.

$$t(0.05) \text{ for } 6 \text{ df} = 2.447$$

$$\text{then, } \underline{\text{lsd}} = 2.447 \times \sqrt{\frac{2 \times 1}{3}} = 2.006$$

The lsd indicates that there is no significant difference between the three means under consideration.

The means that are not significantly different are ranked in one category and are underlined by a bar as shown below.

Means = $\sqrt{\bar{x}}$ 3 9 16

Statistical Analysis of the Data on Viable Plate Counts of
Activated Sludge On Several Media. Plating Experiment VII.

Medium	Activated Sludge From					
	Marlboro		Bernardsville		Morristown	
	(10 ⁶ /ml)	$\bar{x}$	(10 ⁶ /ml)	$\bar{x}$	(10 ⁶ /ml)	$\bar{x}$
ASE (Mar)	48	6.9	122	11	432	20.8
	38	6.2	106	10.3	297	17.2
	32	5.7	69	8.3	327	18.1
			139	11.8	282	16.8
		$\bar{\bar{x}} = 6.26$		10.35		18.25
ASE (Ber)	29	5.4	298	17.3	582	24.1
	26	5.1	336	18.3	638	25.3
	33	5.7	385	19.6	690	26.3
					540	23.2
		$\bar{\bar{x}} = 5.4$		18.4		24.71
ASE (Mor)	74	8.6	170	13.0	219	14.8
	165	12.8	324	18.0	267	16.3
	114	10.7	210	14.5	213	14.6
					377	19.4
		$\bar{\bar{x}} = 10.7$		15.2		16.28
Taylor (Mar)	23	4.8	104	10.2	228	15.1
	21	4.6	148	12.2	219	14.8
	32	5.7	164	12.8	180	13.4
	52	7.2	108	10.4	165	12.8
		$\bar{\bar{x}} = 5.58$		11.4		14.03
Taylor (Ber)	34	5.8	217	14.7	370	19.2
	13	3.6	354	18.8	308	17.5
	24	4.9	171	13.1	209	14.5
	25	5.0	207	14.4	368	19.2
		$\bar{\bar{x}} = 4.8$		15.25		17.6
Taylor (Mor)	60	7.7	305	17.5	387	19.7
	39	6.2	250	15.8	224	15.0
	21	4.6	178	13.3	290	17.0
	35	5.9	244	15.6	289	17.0
		$\bar{\bar{x}} = 6.1$		15.55		17.18
NA	36	6.0	240	15.5	354	18.8
	53	7.3	216	14.7	339	18.4
	48	6.9	210	14.5	260	16.1
	36	6.0	197	14.0	295	17.2
		$\bar{\bar{x}} = 6.55$		14.7		17.63

(a) <u>Analysis of Variance (Transformed Data).</u>		df	MSS	F
Total sum of squares	15923.93	79		
Correction factor	13468.26	1		
Total corrected sum of squares	2455.67	78		
Location sum of squares	1822.48	2	911.24	450
Media sum of squares	490.63	18	27.26	12
Samples (media) (location)	142.56	58	2.23	

All the F values are significantly higher than tabulated

F (2.58) df and F (18.58) df at 0.05 significant level.

Hence the productivity of media are significantly different.

(b) lsd (Least Significant Difference).

$$lsd = 1.98 \times \sqrt{\frac{2 \times 2.23}{4}} = 2.099$$

Summary of ranking viable plate count media in order of effectiveness for activated sludge. Plating Experiment VII.

<u>Activated Sludge</u>								
Marlboro	Medium No.	5	2	4	6	1	7	3
	Mean = $(\bar{x})^2$	23.04	29.16	31.36	37.21	39.69	43.56	114.5
Bernardsville	Medium No.	1	4	7	3	5	6	2
	Mean = $(\bar{x})^2$	108.2	130	216.1	231	231	243.4	338.6
Morristown	Medium No.	4	3	6	7	5	1	2
	Mean = $(\bar{x})^2$	196	265.7	295.8	309.8	309.8	331.2	610

Composition of Culture Media Used in this Investigation.

1. ASE (single strength). One liter of mixed liquor collected from the effluent end of aeration tank was autoclaved for 30 minutes at 121 C. The suspension was filtered through glass wool and the sediment was discarded. The filtrate was made up to one liter with distilled water. Ionagar No.2 was added (0.85%) to the filtrate and autoclaved again for 30 minutes.
2. ASE (double strength). After settling two liters of mixed liquor for 30 minutes one liter of the supernatant was discarded. The remainder was treated as in (1) above.
3. ASE + Soil Extract. Single strength activated sludge extract was prepared as above and mixed with 25% v/v of soil extract. The mixture was autoclaved for about 30 minutes after addition of Ionagar (0.85%) at 121 C.
4. Sewage Agar. One liter of settled sewage was filtered through glass wool. Ionagar No. 2 was added (0.85%) and sterilized for 30 minutes at 121 C.
5. Iron-Peptone Medium.

Proteose Peptone	5 g
Ferrous sulfate	154 mg
Sodium gluconate	90 mg
Ammonium sulfate	100 mg
Tap water	1000 ml

pH 7.0 after autoclaving.

6. Basal Medium (Lochhead and Chase).

Glucose	1 g
Potassium nitrate	0.5 g
Magnesium sulfate	0.2 g
Calcium chloride	0.1 g
Ferric chloride	0.01 g
Distilled water	1000 ml

pH 6.8

7. Basal Medium + Yeast Extract. Basal medium was made as above and 1 g of yeast extract was added per liter of the medium.

8. Taylor's Medium.

Dipotassium hydrogen phosphate	0.5 g
Magnesium sulfate	0.05 g
Sodium chloride	0.01 g
Ferric chloride	0.01 g
Tryptone	0.50 g
Yeast extract	0.50 g
Soil extract	250 ml
Distilled water	750 ml

9. Modified Taylor's Medium. The composition was the same as above except 250 ml of activated sludge extract (single strength) was added instead of the soil extract.

10. Soil Extract. One kg. of soil was suspended in distilled water and autoclaved for 30 minutes at 121 C. The suspension was filtered after addition of a pinch of calcium sulfate. After centrifuging, the supernatant was used for preparation of plating medium by adding 0.85% of Ionagar No. 2 and autoclaving again for 15 minutes at 121 C.

11. Nutrient Agar.

Bacto nutrient agar (Difco)	23 g
Distilled water	1000 ml.

pH 6.8

12. Casitone-Yeast Autolysate Medium.

Casitone	5 g
Yeast autolysate	1.5 g
Distilled water	1000 ml.

pH 7.2

13. Casitone-Glycerol-Yeast Autolysate Medium (CGY).

Casitone	5 g
Glycerol	5 g
Yeast autolysate	1 g

Nutritional Media (Lochhead Series).

14. Basal Medium. (Refer to 6 above).

15. Basal Medium + Yeast Extract (Refer to 7 above).

16. Basal Medium + Amino Acids. Vitamin-free casamino acids (0.4%) were added to basal medium.

17. Basal Medium + Amino Acids + Growth Factors. The following growth factors were added to medium 16 above.

Cysteine	0.05 g
Thiamin	100 ug
Biotin	0.1 ug
Pyridoxin	200 ug
Pantothenic acid	100 ug
Nicotinic acid	100 ug
Riboflavin	200 ug
Inositol	0.05 g

18. Basal Medium + Yeast Extract + Vitamin B₁₂. To medium 15 above vitamin B₁₂ (2 ug per liter) was added.

19. Synthetic Waste Agar.

Glucose	0.5 g
Nutrient broth	0.5 g
Potassium phosphate dibasic	1.0 g
Ammonium phosphate	0.325 g
Sodium chloride	0.050 g
Calcium chloride	0.05 g
Magnesium sulfate	0.05 g
Ferric chloride	0.01 g
Ionagar No. 2	8.5 g
Distilled water	1000 ml

Autoclave for 15 minutes at 121 C and pour plates. (Note: glucose solution should be autoclaved separately).

20. Aromatic Agar Media. To the above synthetic waste medium 500 ppm of a specific aromatic compound was added and autoclaved. In all the following aromatic compounds were used in preparation of the plating media: benzoate, mandelate, phenylacetate, p-hydroxybenzoate, anthranilic acid, benzyl alcohol, o-cresol, m-cresol, p-cresol, catechol and phenol.

21. Basal Medium (Gaudy).

Ammonium chloride	0.5 g
Magnesium sulfate	0.5 g
Calcium chloride	0.01 g
Ferrous sulfate	0.01 g
Manganous sulfate	0.01 g
1 M Phosphate buffer	10 ml
Tap water	100 ml
Make up to 1 liter with distilled water.	
pH	7.2